DNA damage in red blood cells of dab (*Limanda limanda*) and haddock (*Melanogrammus aeglefinus*) as a marker for chemical exposure in the North-East Atlantic

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Abstract

The aim of the current study was to quantify DNA damage in red blood cells from natural populations of dab (*Limanda limanda*) and haddock (*Melanogrammus aeglefinus*) in relation to chemical exposure in their natural habitat. The North-East Atlantic is a highly productive area, which also receives significant amounts of chemicals from landbased, atmospheric and offshore sources. There is reason for concern if hazardous substances in the North-East Atlantic ecosystems affect marine organisms, and a comprehensive set of biomarkers is of fundamental value in developing an integrated monitoring and assessment framework on the health status of marine ecosystems. As part of the international ICON project, dab and haddock were collected, sampled and analyzed for DNA damage in red blood cells, using the Comet assay.

The lack of satisfactory storage methods have until now made the Comet assay less available in field monitoring. An objective was therefore to establish a method for the preservation of blood samples prior to Comet analysis. Another objective was to identify specific DNA lesions caused by oxidative stress.

Dab and haddock were collected and sampled from designated coastal and offshore locations in the North-East Atlantic during cruises from August to late December 2008. Two stations around Iceland were used as reference stations. DNA from fish blood was stored with gradual freezing to -80°C, and direct immersion and storage in lysis buffer for up to 5 weeks. DNA damage was analyzed, using the Comet assay.

The lowest background DNA damage was observed with direct immersion and storage of samples in lysis buffer. Results from the Comet assay clearly indicated that dab from coastal polluted areas (Firth of Forth) and offshore areas with nearby oil and gas activity (Ekofisk) had more DNA damage than dab from less polluted (Iceland) or offshore areas without nearby activity (Dogger Bank). The results were less clear for haddock. It was not possible to identify specific DNA lesions caused by oxidative stress based on available data.

The results clearly support Comet analyses of fish red blood cells as a useful parameter by which to assess environmental stress caused by chemicals, however, care needs to be taken at all steps in sample preparation and analyses.

Contents

1. Introduction

Chemicals are released from natural processes or as a result of human activities and are an essential part of the world we live in. Several of these compounds are hazardous because they are persistent, liable to accumulate in living organisms and/or toxic. Chemicals may be released to soil, air or water, and the aquatic environment is usually the ultimate repository. This means that aquatic organisms in some regions will exist in direct contact with a number of potentially harmful substances. Many chemicals will persist for a period in the aquatic environment even when inputs have been reduced or removed.

The North-East Atlantic includes the North Sea, the Baltic Sea and the Icelandic waters. Intensive fishing pressure has had considerable influence on the ecological balance in this area and is ultimately responsible for decline and collapse of distinct fish stocks (Hislop 1996; Cook et al. 1997; Scott et al. 1999). This course of events has coincided with increasing coastal city populations, global warming, eutrophication and chemical inputs. The latter factors may exert additional pressure on already stressed fish stocks and complex ecosystems. Population density is high in the industrialised and agricultural land masses enclosing the North Sea. This area has received significant amounts of hazardous substances from land-based sources, including metals such as cadmium, mercury and lead and organochlorine compounds such as DDT (dichlorodiphenyltrichloroethane), lindane and PCBs (polychlorinated biphenyls) (OSPAR 2000; Green et al. 2003). Sand and gravel mining, marine traffic and exploitation of offshore oil and gas reserves are activities contributing to elevated concentrations of various metals and organic pollutants. The antifouling paint tributyltin (TBT) has been used extensively in shipping and elevated levels of this compound are found in large harbour areas, with lower levels in areas of less marine traffic (OSPAR 2010). Established levels of persistent organochlorine compounds in several seal species indicate a north-south gradient in the North-East Atlantic, with pollution levels considerably lower in Icelandic waters compared to the North Sea (Luckas et al. 1990). There are relatively few local sources of hazardous substances around Iceland, but long-range transport through oceanic and atmospheric circulation contribute with volatile substances such as mercury, DDT, lindane and PCBs (Vetter et al. 1995; Stephensen et al. 2000). Decreasing inputs of cadmium, mercury and lead have been observed in most areas, but levels of known persistent organochlorines are not decreasing in all areas of the North-East Atlantic (Green et al. 2003; Hylland et al. 2006).

There is serious concern about the continuous release of chemicals into the environment. A collective acknowledgement of problems associated with transboundary pollution has resulted in implementation of national and international measures to reduce production and use of hazardous substances. Decisions by local and regional authorities or industrial environmental managers tend to be made on the basis of predicted influence of separate individual pressures. There is limited knowledge of interactions between co-occuring pressures, and possible consequences of such interactions may be missed. Chemicals in complex mixtures could interact in different ways (i.e. additive, synergistic or antagonistic) to induce responses at different levels of biological organization (Donnelly et al. 1987; Calabrese 1991). Environmental factors could change both the availability and effect of chemicals on marine ecosystems (Hylland 2006). Consequently it is useful to combine chemical analysis with biological methods, to achieve more exact information than attainable with each procedure alone (Donnelly et al. 1987). The Oslo-Paris Commission (OSPAR) has included several techniques to measure the biological effect of chemicals into national and international monitoring activities (JAMP/CEMP). These monitoring activities aim to measure and monitor the quality of the marine environment (Thain et al. 2008). There is an urgent need for an overview of concentrations of hazardous substances in the marine environment and of biological responses caused by exposure to these substances, in other words an integrated monitoring and assessment framework on marine ecosystem health.

The international workshop 'Integrated Assessment of Contaminant Impacts on the North Sea' (ICON) was developed through the ICES/OSPAR working group WKIMON (Thain et al. 2008; Hylland et al. 2010). The aim of this workshop was to assess the health of the North-East Atlantic coastal and offshore ecosystems with regards to hazardous substances and biological effects through an integrated approach. Areas on a wide geographical scale were covered through the designation of sampling stations including Iceland as well as coastal and offshore locations in the North Sea. Icelandic waters are derived from the mixing of water from the central North Atlantic with less polluted waters from the Arctic Ocean (OSPAR 2000). Concentrations of PCBs and PAHs (polycyclic aromatic hydrocarbons) in areas around Iceland are the lowest reported for any oceanographic region (Schulz-Bull et al. 1998). On this basis two areas around Iceland were designated as reference stations; one located just south of the glacier Vatnajökull with no significant sources of anthropogenic pollution nearby and the other in the open ocean west of Reykjavik. The coastal station Firth of Forth is downstream of an industrialised estuary and receives inputs from a wide range of industrial

activities, including heavy metals, hydrocarbons and organochlorine compounds (Elliott and Griffiths 1987). The German Bight is influenced by the rivers Elbe and Weser and has been subject of considerable input of numerous wastes including heavy metals and organochlorines (Hylland et al. 1992; Kammann et al. 2001). The Mecklenburg Bight in the Baltic Sea was previously a repository of industrial waste and sediments in some regions are still very polluted (Wölz et al. 2009). The Dogger Bank is a shallow area about 100 km off the coast in the southern North Sea, with no nearby offshore activity. The Egersund Bank about 100 km off the coast has been used as reference area in previous reports as there is no offshore activity in the vicinity (Hylland et al. 2006). The offshore stations of Dan Field and Ekofisk are the oldest oil fields in operation in the entire North Sea and the Norwegian continental shelf, respectively. The exact composition of hazardous substances from oil and gas exploitation varies greatly between platforms, but PAHs and alkylphenols are always present (Brendehaug et al. 1992; Kingston 1992).

Several requirements must be met when selecting organisms for biomarker studies. Selected organisms should have wide distribution, high abundance and should be relatively stationary. The organisms must obviously be available for sampling. Sufficient uptake of any selected pollutant and survival in polluted environments are also fundamental requirements. An organism that meet these conditions may be used as an indicator organism (Stich et al. 1976).

Common dab (*Limanda limanda*) is a marine flatfish with distribution extending from the Bay of Biscay to the Barents Sea. The species is widespread in the North Sea, in the western Baltic Sea and on the continental shelf of Iceland (Ehrenbaum 1936). It lives in direct contact with sand and silt sediments and is abundant at depths of 20-100 m (Ehrenbaum 1936; Bohl 1957; Henderson 1998). Male and female dab become sexually mature at 1-2 yr of age and 11 cm and 1-3 yr of age and 13 cm, respectively (Bohl 1957), this may have changed in some areas as a result of fishing pressure (Hylland, pers. comm.). Dab spawning grounds are offshore (Henderson 1998) and the spawning presumably takes place near more or less well defined nursery grounds (Daan et al. 1990). The spawning season extends from January to September, following which pelagic eggs and subsequently larvae remain in the water column for weeks (Harding and Nicholls 1987; van der Land 1991). Dab eggs and larvae are found in almost all waters within the geographical range of dab, suggesting considerable gene flow (Henderson 1998) and consequently the presence of "a population of populations" (Levins 1969). No uniform migratory pattern has been established but dab is in general considered to be a

stationary species. The population south of the Dogger Bank has been found restless during spawning time (Damm et al. 1991). Primary food sources of dab are benthic invertebrates such as crustaceans and polychaetes. Molluscs, small echinoderms and fish are also part of the diet (Braber and de Groot 1973). Dab has been used as sentinel species in several monitoring programs (Dethlefsen 2000; Skouras et al. 2003; Rybakovas et al. 2009) and has proved to be suitable as an indicator species and is one of the species recommended by ICES for contaminant-related monitoring.

Haddock (*Melanogrammus aeglefinus*) is a marine gadoid with distribution extending from Portugal to Iceland, Spitsbergen and Novaja Zemlja (Pethon 1998). Haddock prefer deep, warm and saline waters and is absent in the Baltic Sea (Hedger et al. 2004). It lives near mixed sand, clay and gravel bottom and is found at depths of 40-300 m (Pethon 1998; Hedger et al. 2004). In the North Sea sexual maturation occur at 1-4 yr of age and from 21 cm, where male haddock typically mature at slightly younger age than females (Hislop and Shanks 1981). There is apparently no well defined nursery grounds for haddock (Daan et al. 1990), although several spawning areas exist (Saville 1959; Pethon 1998). The spawning season extends from March to June, after which pelagic eggs and subsequent larvae enter the waters (Pethon 1998). In the North-East Atlantic, haddock are abundant around the Faroe Islands, Iceland, west of Scotland, Rockall, the Irish Sea, the Channel through west of Scotland, the North Sea, and the Norwegian coast (Olsen et al. 2010). Despite suggestions of genetically distinct eastern and western populations in the northern North Sea, most views support reports of sufficient gene flow and that there is in fact "a population of populations" (Daan et al. 1990). Seasonal migrations have been documented but the pattern is not clear (Olsen et al. 2010). Primary food sources of haddock are benthic invertebrates such as crustaceans and polychaetes. Bivalves, echinoderms, molluscs, cephalopods and fish are also part of the diet (Albert 1995; Pethon 1998). Haddock has increasingly been used in monitoring activities (Kallenborn et al. 2001; Hylland et al. 2006) and is considered a promising indicator species, in particular due to its wide distribution range in the northern Atlantic.

Evident biological effects following long-term, low-level pollution exposure are generally hard to find due to late manifestation (Hylland 2006). Some effects could lead to reduced health of marine organisms while others are harmless. Damage in germ cells could potentially be passed on to the offspring (Bickham et al. 2000). In cases where an association between a hazardous substance and biological effect is known, the effects could be measured and used as

an indication of unwanted exposure (van der Oost et al. 2003). Biological effects associated with one or several substances, that give a measure of exposure and sometimes of toxic effect, may be used as biomarkers (Peakall and Walker 1994). Effects at higher levels of biological organization are generally preceded by earlier changes in biological processes. This fact enables the use of biomarkers as an early warning signal of unwanted exposure and later of biological effects (Bayne et al. 1975; McIntyre et al. 1978). An improved and comprehensive set of markers is of fundamental value in developing an integrated monitoring and assessment framework.

The need for improved markers has promoted development and increasing use of the Comet assay (single cell gel eletrophoresis) as a technique for measuring DNA damage through assessment of DNA strand breaks or specific DNA lesions caused by oxidative stress. The assay is widely applied in genotoxic studies and often used in human biomonitoring (Valverde and Rojas 2008; Sipinen et al. 2010). The assay has been used to some extent on freshwater fish species in rivers and lakes (Pandrangi et al. 1995; Devaux et al. 1997; Russo et al. 2004). Nevertheless the assay has been scarcely used in studies of pollution-induced DNA damage in marine fish, with a couple of exceptions of natural (Bombail et al. 2001; Brown and Steinert 2004) and cultivated species (Nacci et al. 1996; Belpaeme et al. 1998). The Comet assay is unsuitable with regard to specific exposure because a number of chemicals can induce DNA strand breaks. Nevertheless, the relative simplicity, cost-efficiency and high sensitivity makes it an interesting tool to estimate site-specific DNA damage. Whole blood was selected as sampling tissue, due to the fact that fish red blood cells contain DNA and because of the simple and non-destructive sampling technique. Use of the assay in marine field surveys requires an effective technique for the preservation of sampled tissue. The absence of good storage methods have until now made the Comet assay less available in field monitoring.

The aim of this thesis was to quantify DNA damage in red blood cells from natural populations of dab and haddock in relation to chemical exposure in their natural habitat. This objective involved:

(i) to establish a method for the preservation of blood samples prior to Comet analysis
(ii) to quantify DNA damage in two fish species, dab and haddock, from different locations in the North-East Atlantic, to assess the presence of genotoxic agents
(iii) to identify specific DNA lesions caused by oxidative stress

2. Materials and methods

Descriptions of solutions and media listed in this chapter have been included in Appendix B.

2.1 Vessels

Several cruises were performed under the co-ordination of ICON during the last 5 months of 2008. Three of these cruises were involved in the collection and sampling of dab and haddock for this thesis; RV Walther Herwig III left Bremerhaven on the 29th of August and returned on the 19th of September, FRV Scotia left Aberdeen on the 21st of October and returned on the 28th of October and towards the end of the year RV Walther Herwig III again left Bremerhaven on the 28th of November and returned on the 16th of December.

2.2 Areas

The designated sampling stations (Figure 2.1) were selected to achieve an assessment of pollution impacts in coastal and offshore ecosystems in the North-East Atlantic. A description of the stations is included in chapter 1.

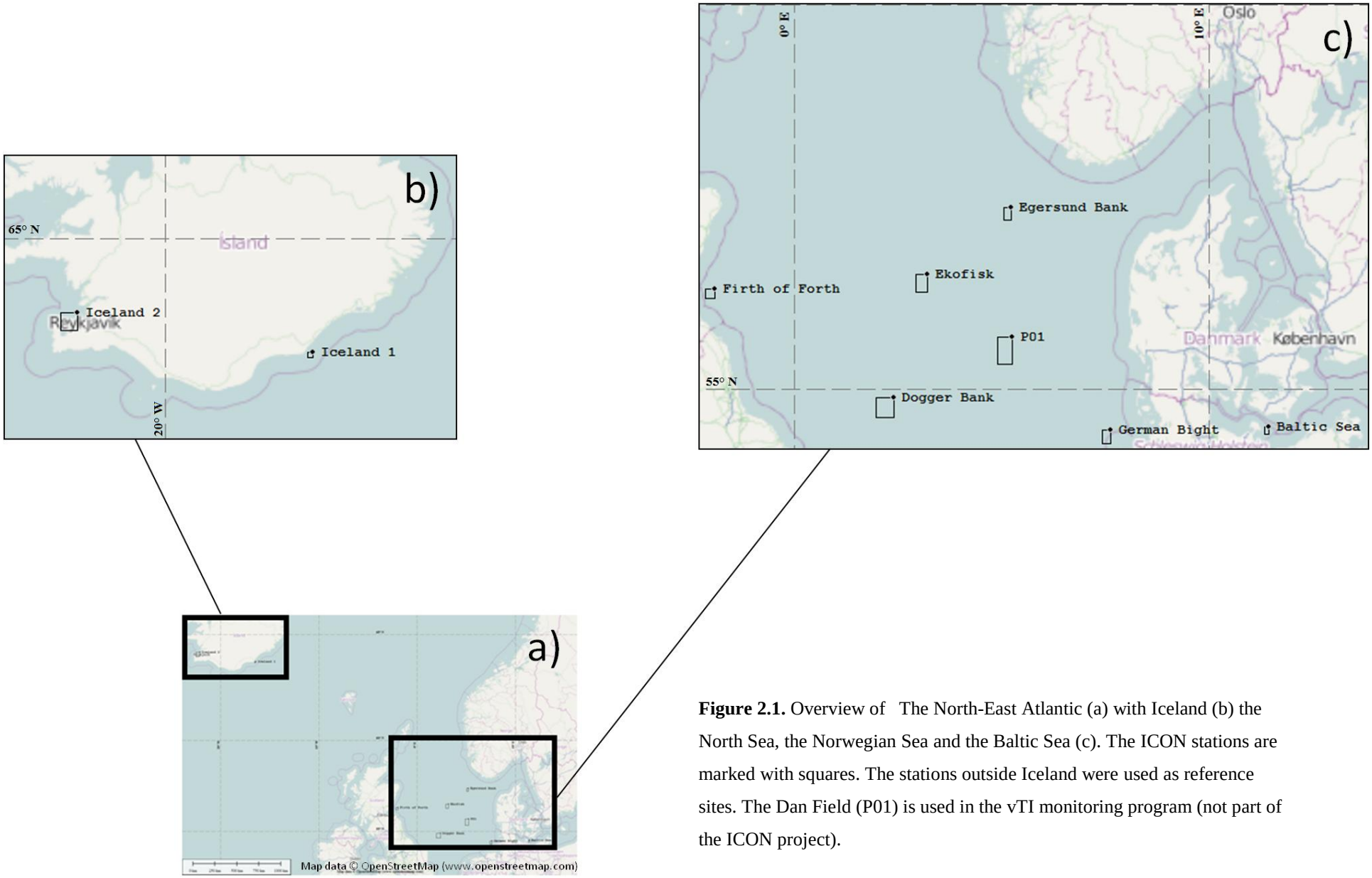

Figure 2.1. Overview of The North-East Atlantic (a) with Iceland (b) the North Sea, the Norwegian Sea and the Baltic Sea (c). The ICON stations are marked with squares. The stations outside Iceland were used as reference sites. The Dan Field (P01) is used in the vTI monitoring program (not part of the ICON project).

2.3 Organisms

Dab (*Limanda limanda*) and haddock (*Melanogrammus aeglefinus*) were used as indicator species. A description of both species is included in chapter 1.

2.4 Catch

A total number of 225 dab and 100 haddock was collected by length stratification (Table 2.1 and 2.2). All collection was carried out by means of bottom trawling and with 30 minutes towing time per haul. The total number of hauls at each station was between 3-6, decided by number and condition of the indicator species in each haul.

Table 2.1. Male dab collected at the designated stations. Age, length, body weight, liver weight and gonad weight are given as median and quartiles. Liver weight and gonad weight was not measured at the Dan Field.

Station	♂	Age	Length (cm)	Body wt (g)	Liver wt (g)	Gonad wt (g)
Iceland 1	9	4.0; 3.0 - 4.5	25.0; 23.0 - 26.0	146.0; 117.0 - 165.0	2.1; 1.4 - 2.6	0.5; 0.4 - 0.6
Iceland 2	9	3.0; 3.0 - 3.0	22.0; 22.0 - 23.5	119.0; 111.5 - 156.0	1.6; 0.9 - 2.5	0.4; 0.2 - 0.7
Firth of Forth	5	2.0; 2.0 - 2.5	21.0; 20.5 - 21.0	91.0; 87.0 - 94.0	1.3; 1.1 - 1.7	1.0; 0.7 - 1.4
German Bight	1	2.0; 2.0 - 2.0	20.0; 20.0 - 20.0	110.0; 90.3 - 122.0	1.2; 1.2 - 1.2	0.2; 0.2 - 0.2
Baltic Sea	11	2.0; 2.0 - 3.0	24.0; 23.0 - 24.0	132.0; 128.0 - 156.0	1.5; 1.1 - 1.8	2.3; 2.1 - 2.5
Dogger Bank	0	-	-	-	-	-
Egersund Bank	11	4.0; 3.0 - 4.0	22.0; 21.0 - 23.0	84.0; 77.0 - 87.0	1.1; 0.9 - 1.2	1.1 1.0 - 1.4
Dan Field	11	3.0; 2.0 - 3.0	21.5; 20.8 - 23.3	101.5; 83.0 - 131.5	-	-
Ekofisk	2	2.5; 2.0 - 3.0	22.5; 21.0 - 24.0	126.5; 123.0 - 130.0	1.3; 0.8 - 1.8	0.4; 0.2 - 0.5

Table 2.2. Female dab collected at the designated stations. Age, length, body weight, liver weight and gonad weight are given as median and quartiles. Liver weight and gonad weight was not measured at the Dan Field.

Station	♀	Age	Length (cm)	Body wt (g)	Liver wt (g)	Gonad wt (g)
Iceland 1	16	4.0; 2.0 - 4.0	23.5; 22.0 - 25.0	117.5; 99.3 - 158.3	1.5; 1.2 - 2.2	1.3; 1.0 - 1.7
Iceland 2	16	3.0; 2.5 - 4.0	24.0; 22.0 - 25.8	145.5; 114.5 - 178.3	1.6; 1.2 - 2.2	0.8; 0.5 - 1.2
Firth of Forth	20	3.0; 2.3 - 3.8	22.0; 21.0 - 22.0	109.0; 92.0 - 125.3	1.6; 1.1 - 1.8	1.2; 0.8 - 1.6
German Bight	24	2.0; 2.0 - 3.0	22.0; 20.3 - 23.0	110.0; 90.3 - 122.0	2.2; 1.7 - 3.1	1.1; 1.0 - 1.5
Baltic Sea	14	2.0; 2.0 - 2.0	23.0; 23.0 - 24.3	142.5; 120.5 - 150.0	2.2; 1.5 - 2.9	2.6; 1.5 - 4.4
Dogger Bank	25	3.0; 2.0 - 3.0	22.0; 21.0 - 23.0	114.0; 91.5 - 129.0	1.3; 1.0 - 2.1	1.1; 0.9 - 1.3
Egersund Bank	14	5.0; 3.8 - 5.0	22.0; 21.0 - 23.0	93.5; 82.3 - 109.5	1.1; 0.8 - 1.6	1.4; 1.2 - 1.4
Dan Field	14	4.0; 3.0 - 4.0	23.0; 22.0 - 25.0	121.0; 103.0 - 163.0	-	-
Ekofisk	23	3.0; 2.0 - 3.0	22.0; 21.0 - 23.0	99.0; 86.0 - 124.0	1.4; 1.1 - 1.6	1.3; 1.0 - 1.6

Table 2.3. Male haddock collected at the designated stations. Age, length, body weight, liver weight and gonad weight are given as median and quartiles.

Station	♂	Age (yrs)	Length (cm)	Body wt (g)	Liver wt (g)	Gonad wt
Iceland 1	16	3.0; 3.0 - 4.0	39.0; 37.0 - 41.0	532.0; 411.0 - 650.5	23.9; 13.3 - 35.9	0.6; 0.8 - 2.6
Iceland 2	15	3.0; 2.0 - 5.0	37.0; 33.0 - 39.0	491.0; 368.0 - 543.0	14.2; 6.8 - 20.4	0.5; 0.3 - 0.6
Firth of Forth	13	3.0; 2.0 - 3.0	27.0; 26.0 - 29.5	204.0; 181.0 - 266.0	9.4; 6.5 - 14.6	0.4; 0.4 - 0.6
Egersund Bank	17	1.0; 1.0 - 2.5	30.0; 28.5 - 35.0	257.0; 222.0 - 421.0	14.9; 11.0 - 23.2	0.8; 0.7 - 3.3

Table 2.4. Female haddock collected at the designated stations. Age, length, body weight, liver weight and gonad weight are given as median and quartiles.

Station	♀	Age (yrs)	Length (cm)	Body wt (g)	Liver wt (g)	Gonad wt
Iceland 1	9	3.0; 3.0 - 3.0	38.0; 40.0 - 41.0	662.0; 564.5 - 716.5	28.0; 22.2 - 36.4	2.6; 2.5 - 3.2
Iceland 2	10	3.0; 2.0 - 5.0	36.0; 32.8 - 40.3	479.0; 331.3 - 604.8	13.0; 4.8 - 22.3	1.9; 1.0 - 3.1
Firth of Forth	12	3.0; 2.0 - 3.0	28.0; 25.6 - 33.0	283.5; 229.8 - 347.0	12.3; 9.8 - 16.4	1.6; 1.4 - 2.5
Egersund Bank	8	1.0; 1.0 - 1.0	31.0; 28.5 - 31.0	246.5; 206.5 - 271.8	18.1; 12.9 - 21.6	0.9; 0.6 - 1.5

Environmental parameters, including temperature, salinity and oxygen saturation, were recorded during trawling. Measurements were taken for several stations within each area. Average data and coordinates are presented for each station (Table 2.3).

Table 2.5. Hydrographical measures of temperature (T), salinity (S) and oxygen saturation (O_2) at bottom depth of the respective stations. O_2 was not measured at the Egersund Bank.

Date	Area	Depth (m)	T (°C)	S (PSU)	O_2 (%)	Latitude area	Longitude area
05.09.08	Iceland 1	54-83	8.6	35.2	84.8	63°46.07N	16°25.87W
06.09.08	Iceland 2	30-51	11.3	34.6	68.6	64°07.98N	22°17.40W
13.09.08	Firth of Forth	47-51	12.7	34.8	90.1	56°18.82N	02°01.44W
16.09.08	German Bight	21-38	17.3	33.8	92.0	54°18.04N	07°30.04E
06.12.08	Baltic Sea	18-20	6.7	17.7	93.3	54°20.99N	11°24.50E
01.09.08	Dogger Bank	16-24	16.5	34.5	97.1	54°45.26N	02°12.42E
26.10.08	Egersund Bank	53-78	10.6	34.5	-	57°36.35N	05°12.39E
31.08.08	Dan Field	38-41	10.3	34.7	73.6	55°24.76N	05°06.10E
14.09.08	Ekofisk	66-71	7.2	35.0	80.4	56°30.51N	03°05.02E

On board RV Walther Herwig III, the trawl was emptied in a funnel passing through the trawl deck to a sorting hall. On board FRV Scotia, the catch was released directly on trawl deck. Dab and haddock were selected by size and condition, and kept alive by rapid transfer to large tanks with sea ice and running sea water. Fish were transferred in suitable groups to smaller tanks in the sampling lab.

2.5 Sampling

Each fish was treated with concussive shock prior to sampling. Body length, total weight, liver weight, gonad weight and sex were recorded (Table 2.1-2.4). Blood was extracted from the caudal vein with a syringe using a 0.7 x 30 mm cannula. The syringe was precoated in heparin to avoid coagulation. Approximately 100 µl of blood was transferred to respective Eppendorf tubes containing 900 µl phosphate buffer solution (PBS) and 10 mM etylenediaminetetraacetic acid (EDTA). The diluted blood was kept on ice for subsequent processing.

2.6 Determination of age

Age determination of dab and haddock were done at the von Thünen Institut in Cuxhaven, Germany, as part of the ICON project (Hylland et al. 2010).

2.7 Liver and gonad somatic indexes

Liver somatic index (LSI) is an indirect measure of the food availability of fish. Large intake of food could result in elevated lipid content and size increase of the liver (Tyler and Dunn 1976). LSI gives percent liver weight of total weight and was calculated as follows; LSI = liver weight/body weight × 100 (Slooff et al. 1983).

Gonad somatic index (GSI) is an indicator of somatic and reproductive investment of mature fish (Tyler and Dunn 1976; Malavasi et al. 2004). GSI gives percent gonad weight of total weight and was calculated as follows; GSI = gonad weight/body weight × 100 (Morley et al. 2010).

2.8 Sample storages

2.8.1 Pilot

Prior to departure of the first cruise, several freezing techniques were tested in the lab to find optimal storage conditions through treatment of blood from flounder (*Platichthys flesus*). The techniques were; freezing directly at -80°C, freezing at -80°C using the freezing device 'Mr. Frosty' (5100 Cryo 1°C Freezing Container, Nalgene Labware), snap-freeze in liquid nitrogen

(-196°C), freezing at -20°C using a polystyrene box and direct immersion in lysis buffer (Section 2.8.3). The 'Mr. Frosty' freezing unit has a cooling rate of -1°C per minute until final temperature is reached. The procedures with freezing directly at -80°C and snap-freeze in liquid nitrogen were both discarded after visual assessment (massive DNA damage). Procedures with freezing at -80°C using 'Mr. Frosty', freezing at -20°C using a polystyrene box and direct immersion in lysis buffer were all three considered satisfactory in the pilot study (results not shown). Further testing with these techniques took place during the first cruise.

2.8.2 Freezing

Subsequent processing of the diluted blood in the field took place as follows. All tubes used for storage of sampling material were marked with station, species, number and tissue. 50 µl of the diluted blood was transferred from respective Eppendorf tubes to corresponding Cryo tubes containing 450 µl of freezing solution. All Cryo tubes were lined up inside the inner chamber of 'Mr. Frosty'. The inner chamber was designed for 18 Cryo tubes. This capacity was increased by arranging the remaining tubes (19-25) in the upper part of the inner chamber. 250 ml of isopropanol was added to the surrounding chamber and the unit was placed in a freezer holding -80°C.

Another 50 µl of the diluted blood was transferred from respective Eppendorf tubes to corresponding Cryo tubes containing 450 µl of freezing solution. The Cryo tubes were lined up in a rack inside a polystyrene box and placed at -20°C.

Frozen samples from -20°C were placed in the -80°C freezer before transportation to the lab in Norway. Most frozen samples were subsequently immersed in liquid nitrogen and carried in a cryoshipper (CXR500 Dry Shipper, Taylor-Wharton). Frozen samples from the Egersund Bank were transported on dry ice.

The technique of freezing samples at -20°C using a polystyrene box was discarded after visual assessment of samples from the first cruise. Results presented in this thesis are generated with the technique of freezing at -80°C using 'Mr. Frosty'.

2.8.3 Lysis

Subsequent processing of the diluted blood in the field took place as follows. Lysis buffer was prepared and set aside at 4°C. Agarose (0.75%) was dissolveed in PBS + EDTA using a microwave oven. The agarose was then kept fluid at a temperature of approximately 37°C.

Five µl of the initially diluted blood was transferred from respective Eppendorf tubes to corresponding Eppendorf tubes containing 195 µl PBS + EDTA. The diluted blood was kept on ice.

27 µl of the agarose was transferred to respective Eppendorf tubes and added 3 µl of the diluted blood. 10 µl of this mixture was fixed on Gelbond® films on a metal plate (cold surface). The films were marked with station, species, number and tissue and placed in lysis buffer over a period ranging from 10-26 days for the different stations.

2.9 Comet analysis

2.9.1 Treatment of fish blood

The tubes with frozen suspension were warmed in the hand until it was possible to transfer the suspension into tubes with 10 ml PBS + EDTA on ice. After complete thawing in buffer, the tubes were directly centrifuged at 400 G for 10 minutes at 4°C. After removal of the supernatant, remaining pellets were resuspended in 10 ml PBS + EDTA.

2.9.2 Comet assay

A Comet assay protocol based on Singh et al. (1988) was used, with some modifications (Collins et al. 1993; Collins 2004). Further processing of the thawed freezing samples described in section 2.8.2 and 2.9.1 was as described for *initial*, *enzyme treatment* and *advanced procedure* (see below). Processing of the films described in section 2.8.3 was as described for *advanced procedure* (see below). Every step was performed in dim light.

Initial procedure

The initial Comet procedure was carried out as described in section 2.8.3, with some additional steps. Separate species samples of dab and haddock were randomized with codes to

secure an analysis with no knowledge as to the origin of the samples. Four replicates (4 x 7.5 µl) of each sample were fixed on two separate films; one designated for treatment with Fpg and one used as reference (buffer incubation without enzyme). The films were immersed in lysis buffer over night.

Enzyme treatment procedure

The films were rinsed in dH_2O and immersed in enzyme reaction buffer for 10 minutes at 4°C. The enzyme reaction buffer was then replaced with fresh enzyme reaction buffer and the films were kept immersed for another 50 minutes. Fpg extract was thawed and added to heated enzyme reaction buffer. The films were placed in heated enzyme reaction buffer both with and without (control) enzyme and kept at 37°C for 1 hour.

Advanced procedure

The films were rinsed in dH_2O and unwinding of DNA was accomplished by placing the films in electrophoresis buffer (pH 13.2) for 5 minutes at 4°C. The electrophoresis buffer was then replaced with fresh electrophoresis buffer and the films were kept immersed for another 35 minutes for more unwinding of DNA.

Gel electrophoresis was performed in electrophoresis buffer at 8°C for 20 minutes at 22 V and approximately 650 mA, with an approximate voltage drop of 1.1 V/cm over the platform.

The films were neutralised for 5 minutes in neutralising buffer at room temperature. The neutralising buffer was then replaced with fresh neutralising buffer and the films were kept immersed for another 5 minutes.

After rinsing in dH_2O for 1 minute, the films were placed in absolute ethanol for 5 minutes. The ethanol was then replaced with > 70% ethanol and the films were kept immersed for ≥ 1.5 hour for fixation.

After fixation the films were allowed to dry and eventually stored in the dark at room temperature (could be stored up to several months).

The films (nucleoids) were stained using 20 µl of the dye SYBR® Gold (1000X stock in DMSO) in 25 ml TE-buffer per film for 20 minutes. This dye offers some advantages in the

context of sensitivity and quenching. SYBR® Gold binds to DNA and the films should be allowed to rest for at least one hour under moist and dark conditions at 8°C with respect to a possible delay in this event.

2.9.3 Scoring

Olympus BX51 (Japan) microscope with Olympus Burner (Osram Mercury Short-Arc HBO® 100 W/2 lamp) and A312f camera from Basler Vision Technologies (Germany) was used as well as the 'Comet assay IV' software for image analysis, from Perceptive Instruments (UK). SYBR® Gold binding to DNA (section 2.9.2) results in emission of fluorescence, thereby visualising the nucleoids. Nucleoid density and background was considered in line with standards for the Comet assay and films (gels) with high density or high background were not used. A set of guidelines were followed as to selection of nucleoids during scoring; there was no selection of nucleoids in close vicinity of each other (overlapping or potentially overlapping if they had been damaged), too close to the edge of the image (where any potential overlapping with nucleoids outside the image could not be determined), abnormal cells (tail in wrong direction, abnormal shape of nucleoid), and cells close to foreign objects. These guidelines should as far as possible prevent bias in a procedure where the operator has to select the nucleoids for analysis. The system calculates the total intensity, head intensity (% head DNA), tail intensity (% tail DNA) and tail moment for each nucleoid (comet). The scoring is preferably made without manual interference, however, obvious errors with respect to marking of the comets was corrected when this could be done visually. The scoring was performed with no knowledge as to the origin of the samples. For additional storage, the films were dried and stored under dry and dark conditions at room temperature after the scoring was concluded. The results were presented as tail DNA as it is considered to be the most suitable parameter (Belpaeme et al. 1998; Kumaravel and Jha 2006).

2.10 PAH metabolites

PAH metabolites in bile of dab and haddock were analyzed by Ulrike Kammann at the von Thünen Institut in Hamburg, Germany, as part of the ICON project (Hylland et al. 2010).

2.11 Statistical analysis of data

All data was checked for homogeneity of variance using Levenes test (Levene 1960). In cases with variance equality, one-way analysis of variance (ANOVA) was carried out (Zar 2010). Student's t test was used to test means between two groups (Zar 2010). Tukey-Kramer test was used to test means between more than two groups (Zar 2010). In cases of variance inequality the data was $\log_{10}$-transformed and checked for homogeneity of variance again. In cases of still variance inequality, parameters were tested using non-parametric tests. Mann-Whitney test was used to test means between two groups (Zar 2010). Kruskal-Wallis test and Dunn posthoc test were used to test means between more than two groups (Zar 2010). For all statistical results, a probability of $p < 0.05$ was considered significant (Cowles and Davis 1982; Zar 2010). Statistical analyses were carried out using JMP® 8.0.2.2 (SAS Institute Inc.) and GraphPad Prism 5.00 for Windows (GraphPad Software, Inc.).

All data (including tables) is presented as median and quartiles. A graphical presentation of data is by box plots with median, quartiles and 10% - 90% percentile (whiskers). Scatterplots were used to illustrate the degree of correlation between two variables.

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3. Results

3.1 Method development

3.1.1 Preservation of samples

Tail DNA of samples with gradual freezing to -80°C and immersion in lysis buffer was compared for stations with sufficient data on both storage techniques.

Tail DNA of dab samples was significantly different between storage techniques (Figure 3.1). Tail DNA of samples with gradual freezing was significantly higher compared to samples with direct immersion in lysis buffer for Iceland 1 (31.9; 9-2-44.7 and 2.0; 1.0-7.9) (Student's t test, $p < 0.0001$, $N \geq 13$), Iceland 2 (14.8; 5.5-26.3 and 1.6; 1.2-1.9) (Mann-Whitney, $p < 0.0001$, $N \geq 17$) and the Dogger Bank (15.8; 1.3-24.5 and 2.0; 1.4-2.6) (Mann-Whitney, $p = 0.04$, $N \geq 15$). Tail DNA of samples with gradual freezing was significantly lower compared to samples with direct immersion in lysis buffer for the Firth of Forth (12.6; 4.2-25.1 and 28.9; 21.5-35.6) (Student's t test, $p = 0.001$, $N \geq 17$).

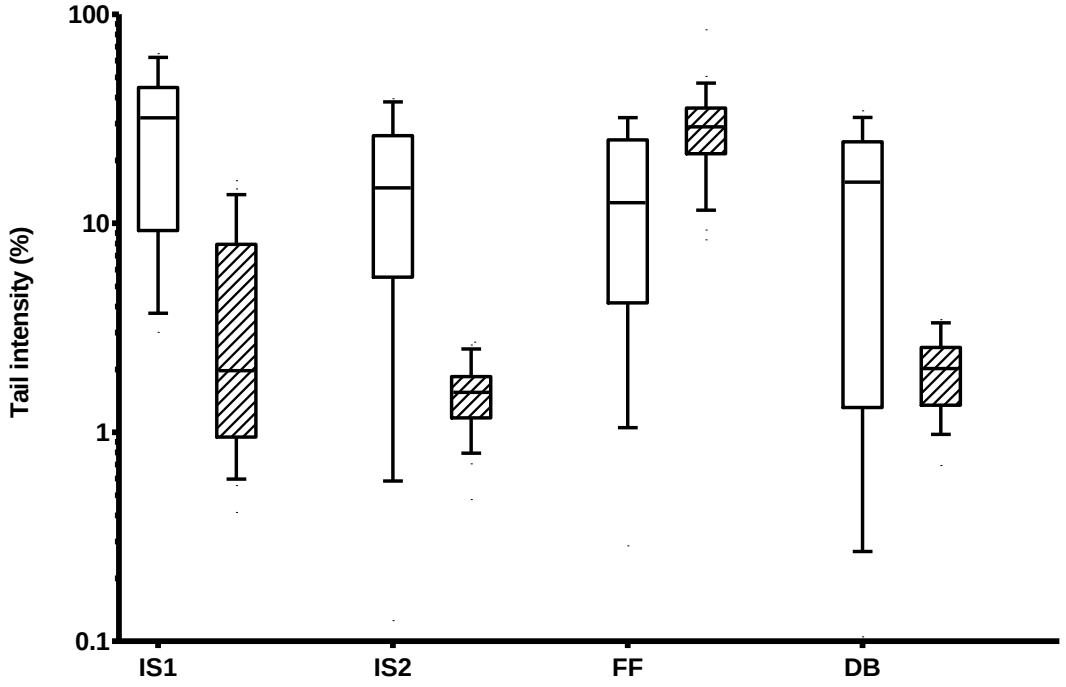

Figure 3.1. Tail DNA of dab with gradual freezing (blank) and direct immersion in lysis buffer (shaded) presented as median, quartiles and 10% - 90% percentile. Tail DNA of samples with gradual freezing was significantly different compared to samples with direct immersion in lysis buffer for Iceland 1 (Student's test, $p < 0.0001$, $N \geq 13$), Iceland 2 (Mann-Whitney, $p < 0.0001$, $N \geq 17$), the Firth of Forth (Student's t test, $p = 0.001$, $N \geq 17$) and the Dogger Bank (Mann-Whitney, $p = 0.04$, $N \geq 15$).

Tail DNA of haddock samples was significantly different between storage techniques (Figure 3.2). Tail DNA of samples with gradual freezing was significantly lower compared to samples with direct immersion in lysis buffer for Iceland 1 (12.8; 9.1-21.3 and 46.7; 8.2-60.1) (Mann-Whitney, $p = 0.04$, $N \geq 20$). Tail DNA of samples with gradual freezing was significantly higher compared to samples with direct immersion in lysis buffer for the Firth of Forth (12.0; 5.1-25.8 and 2.8; 0.8-5.9) (Student's t test, $p = 0.001$, $N \geq 20$) (Figure 3.2).

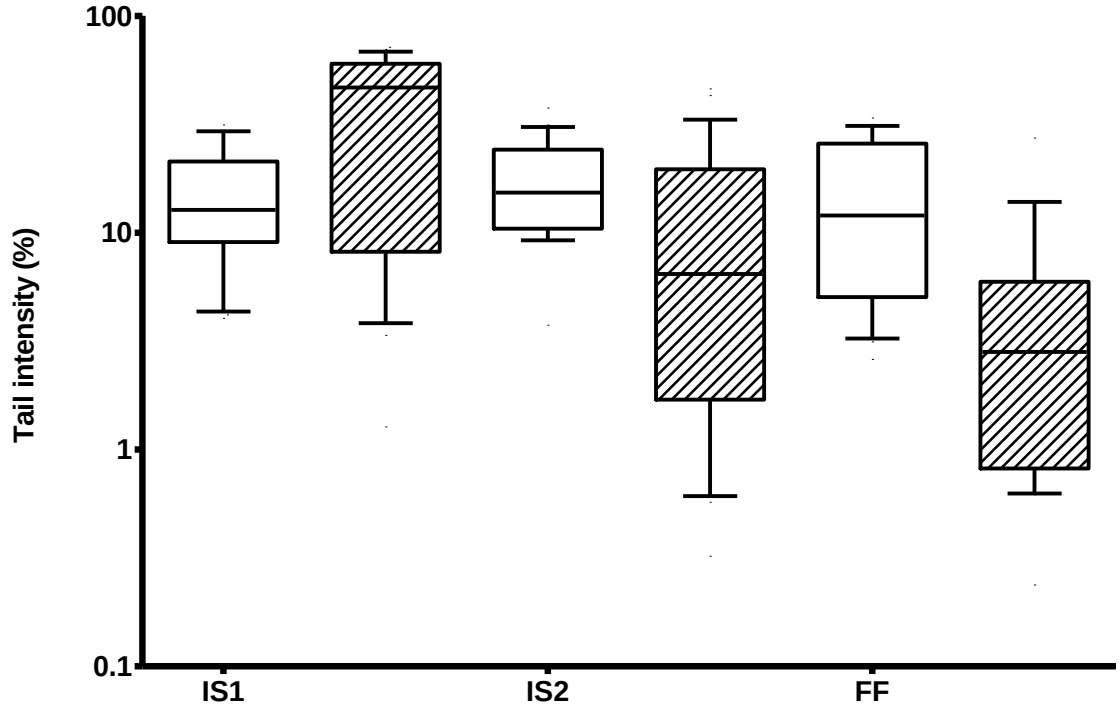

Figure 3.2. Tail DNA of haddock with gradual freezing (blank) and direct immersion in lysis buffer (shaded) presented as median, quartiles and 10% - 90% percentile. Tail DNA of samples with gradual freezing was significantly different compared to samples with direct immersion in lysis buffer for Iceland 1 (Mann-Whitney, $p = 0.04$, $N \geq 20$) and the Firth of Forth (Student's t test, $p \leq 0.0001$, $N \geq 20$).

3.1.2 Oxidative stress

Tail DNA of dab and haddock samples with gradual freezing was not analyzed statistically as a consequence of the analysis and results in section 3.2.1. Tail DNA median values were not apparently different between areas. Tail DNA median values of samples treated with Fpg were higher compared to the reference samples (buffer incubation with no enzyme).

3. 2 Condition and maturation status

Liver somatic index (LSI) between stations was analysed separately for male and female dab (Table 3.1). For males LSI was not significantly different between Iceland 1, Iceland 2, the Firth of Forth, the Baltic Sea, the Egersund Bank and Ekofisk (ANOVA, $p = 0.3$). For females LSI was significantly higher for the German Bight compared to Iceland 1, Iceland 2, the Firth of Forth, the Dogger Bank, the Egersund Bank and Ekofisk (Tukey-Kramer, $p \leq 0.009$). LSI was not significantly different between the German Bight and the Baltic Sea or between the remaining stations (Tukey-Kramer, $p \geq 0.2$).

Gonad somatic index (GSI) between stations was analysed separately for male and female dab (Table 3.1). For males GSI was significantly higher for the Egersund Bank and the Baltic Sea compared to Iceland 1 and Iceland 2 (Tukey-Kramer, $p \leq 0.03$). For females GSI was significantly higher for the Egersund Bank and the Baltic Sea compared to Iceland 2 and the Dogger Bank (Dunn, $p < 0.05$). GSI was also significantly lower for Iceland 2 compared to Iceland 1, the Firth of Forth, Ekofisk and the German Bight (Dunn, $p < 0.05$). GSI was not significantly different between Iceland 1, the Firth of Forth, the German Bight, the Dogger Bank and Ekofisk (Dunn, $p > 0.05$).

Table 3.1. Dab sampled at the designated stations, sorted by sex; male (♂) or female (♀). Liver somatic index (LSI) and gonad somatic index (GSI) data is given as median and quartiles. Data not marked by the same letter were significantly different (Tukey-Kramer, $p \leq 0.02$, Dunn, $p < 0.05$). No liver or gonad data was registered for the Dan Field.

Area	♂	♀	**LSI ♂**	**LSI ♀**	**GSI ♂**	**GSI ♀**
Iceland 1	9	16	1.3; 1.0 - 1.7 [a]	1.4; 1.1 - 1.6 [b]	0.3; 0.3 - 0.5 [b]	1.0; 0.8 - 1.3 [b]
Iceland 2	9	16	1.4; 0.8 - 1.6 [a]	1.1; 0.9 - 1.5 [b]	0.3; 0.2 - 0.5 [b]	0.6; 0.5 - 0.7 [c]
Firth of Forth	5	20	1.4; 1.2 - 1.9 [a]	1.3; 1.1 - 1.8 [b]	0.7; 0.6 - 0.8 [b]	1.0; 0.9 - 1.4 [b]
German Bight	0	24	-	1.9; 1.5 - 3.0 [a]	-	1.0; 0.9 - 1.3 [b]
Baltic Sea	≥10	14	1.1; 0.9 - 1.2 [a]	1.6; 1.0 - 2.0 [ab]	1.8; 1.4 - 2.3 [a]	1.8; 1.2 - 3.0 [a]
Dogger Bank	0	≥20	-	1.3; 1.0 - 1.8 [b]	-	1.0; 0.9 - 1.1 [b]
Egersund Bank	11	≥13	1.2; 1.2 - 1.5 [a]	1.4; 0.8 - 1.6 [b]	1.5; 1.2 - 1.6 [a]	1.4; 1.2 - 1.6 [a]
Ekofisk	2	23	1.1; 0.7 - 1.4 [a]	1.2; 1.1 - 1.7 [b]	0.3; 0.2 - 0.4 [b]	1.2; 1.0 - 1.4 [b]

LSI between stations was analysed separately for male and female haddock (Table 3.2). For males LSI was significantly higher for the Egersund Bank compared to Iceland 1 and Iceland 2 (Tukey-Kramer, $p \leq 0.01$). LSI was also significantly lower for Iceland 2 compared to Iceland 1 and the Firth of Forth (Tukey-Kramer, $p \leq 0.01$). LSI was not significantly different between the Egersund Bank and the Firth of Forth or between the Firth of Forth and Iceland 1 (Tukey-Kramer, $p \geq 0.2$). For females LSI was significantly higher for the Egersund Bank

compared to Iceland 1, Iceland 2 and the Firth of Forth (Tukey-Kramer, $p \leq 0.01$). LSI was not significantly different between Iceland 1, Iceland 2 and the Firth of Forth (Tukey-Kramer, $p \geq 0.4$).

GSI between stations was analysed separately for female and male haddock (Table 3.2). For males GSI of haddock was significantly higher for the Egersund Bank compared to Iceland 1 and Iceland 2 (Dunn, $p < 0.05$). LSI was also significantly higher for the Firth of Forth compared to Iceland 2 (Dunn, $p < 0.05$). LSI was not significantly different between Iceland 1 and the Firth of Forth (Dunn, $p > 0.05$). For females GSI was significantly higher for the Firth of Forth compared to Iceland 1, Iceland 2 and the Egersund Bank (Tukey-Kramer, $p \leq 0.04$). GSI was not significantly different between Iceland 1, Iceland 2 and the Egersund Bank (Tukey-Kramer, $p \geq 0.8$).

Table 3.2. Haddock sampled at the designated stations, sorted by sex; male (♂) or female (♀). Liver somatic index (LSI) and gonad somatic index (GSI) data is given as median and quartiles. Data not marked by the same letter were significantly different (Tukey-Kramer, $p \leq 0.04$, Dunn, $p < 0.05$).

Area	♂	♀	**LSI ♂**	**LSI ♀**	**GSI ♂**	**GSI ♀**
Iceland 1	≥15	≥9	3.8; 3.1 - 5.6 [b]	4.7; 3.5 - 5.7 [b]	0.1; 0.1 - 0.1 [c]	0.4; 0.3 - 0.5 [b]
Iceland 2	≥15	≥9	2.9; 1.4 - 3.4 [c]	2.3; 1.4 - 4.4 [b]	0.1; 0.1 - 0.2 [bc]	0.4; 0.4 - 0.6 [b]
Firth of Forth	13	12	4.4; 3.5 - 6.0 [ab]	4.5; 3.6 - 5.5 [b]	0.2; 0.2 - 0.3 [ac]	0.6; 0.5 - 0.7 [a]
Egersund Bank	≥16	8	5.6; 5.0 - 6.3 [a]	7.6; 6.0 - 8.6 [a]	0.4; 0.2 - 0.7 [a]	0.4; 0.3 - 0.5 [b]

3.3 DNA damage

3.3.1 Comet

Tail DNA of dab samples was significantly different between areas (Figure 3.3). Tail DNA of samples was significantly higher for the Firth of Forth (28.9; 21.5-35.6) and Ekofisk (11.9; 5.2-19.6) compared to Iceland 1 (2.0; 1.0-7.9), Iceland 2 (1.6; 1.2-1.9) and the Dogger Bank (2.0; 1.4-2.6) (Dunn, $p < 0.05$, $N \geq 15$). Tail DNA of samples was also significantly higher for the Firth of Forth compared to the German Bight (6.4; 1.6-9.1) and the Dan Field (3.3; 2.3-6.1) (Dunn, $p < 0.05$, $N \geq 18$). Tail DNA of samples was not significantly different between Iceland 1, Iceland 2, the German Bight, the Dogger Bank and the Dan Field (Dunn, $p > 0.05$, $N \geq 15$). Tail DNA of samples was not significantly different between the Firth of Forth and Ekofisk, or between Ekofisk, the German Bight and the Dan Field (Dunn, $p > 0.05$, $N \geq 18$).

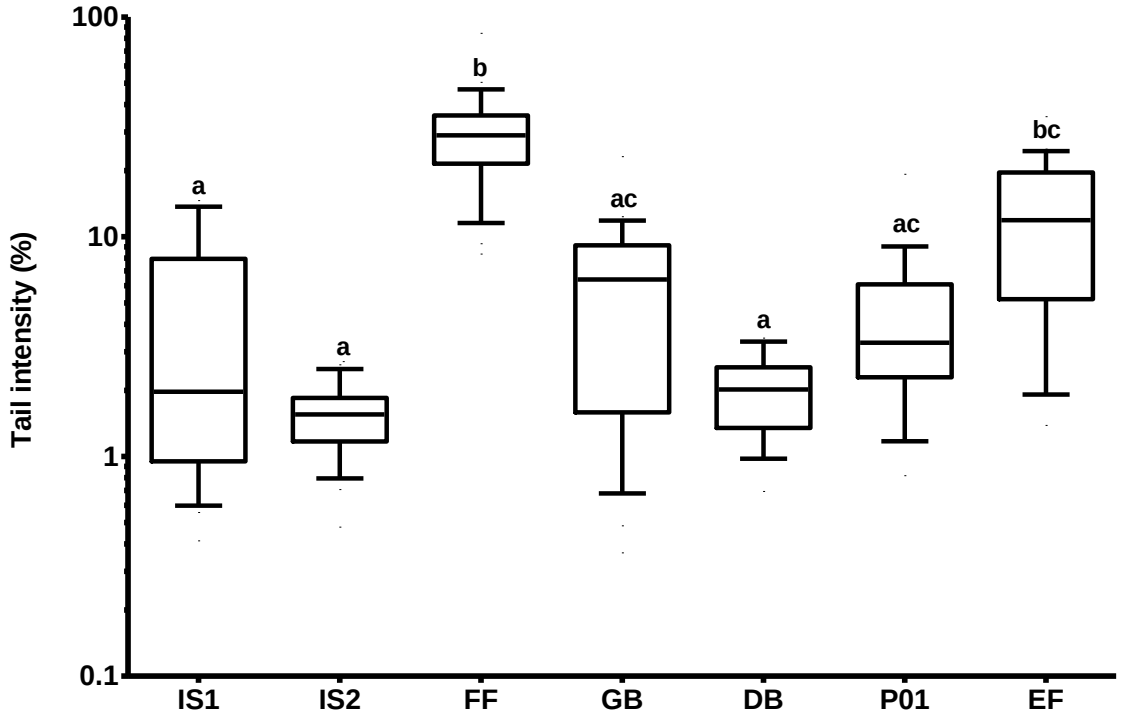

Figure 3.3. Tail DNA for dab with direct immersion in lysis buffer presented as median, quartiles and 10% - 90% percentile. Locations not marked by the same letter was significantly different (Dunn, $p < 0.05$, $N \geq 15$).

Tail DNA of haddock samples was significantly different between areas (Figure 3.4). Tail DNA of samples were higher for Iceland 1 (46.7; 8.2-60.1) compared to Iceland 2 (6.5; 1.7-19.6) (Tukey-Kramer, $p = 0.001$, $N \geq 22$) and the Firth of Forth (2.8; 0.8-5.9) (Tukey-Kramer, $p < 0.0001$, $N \geq 20$). Tail DNA of samples was not significantly different between Iceland 2 and the Firth of Forth (Tukey-Kramer, $p > 0.1$, $N \geq 20$).

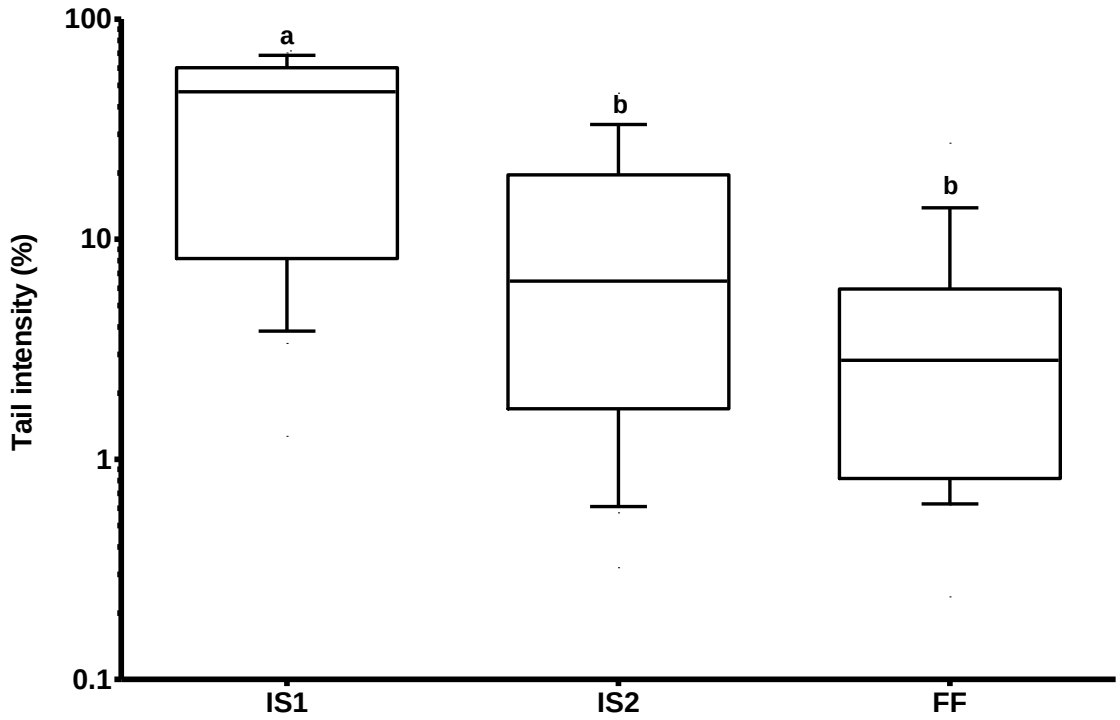

Figure 3.4. Tail DNA for haddock with direct immersion in lysis buffer presented as median, quartiles and 10% - 90% percentile. Locations not marked by the same letter was significantly different (Tukey-Kramer, $p \leq 0.001$, $N \geq 20$).

3.3.2 PAH exposure

Scatterplots illustrating correlation between DNA damage and 1-hydroxypyrene (Appendix A) in bile of dab and haddock.

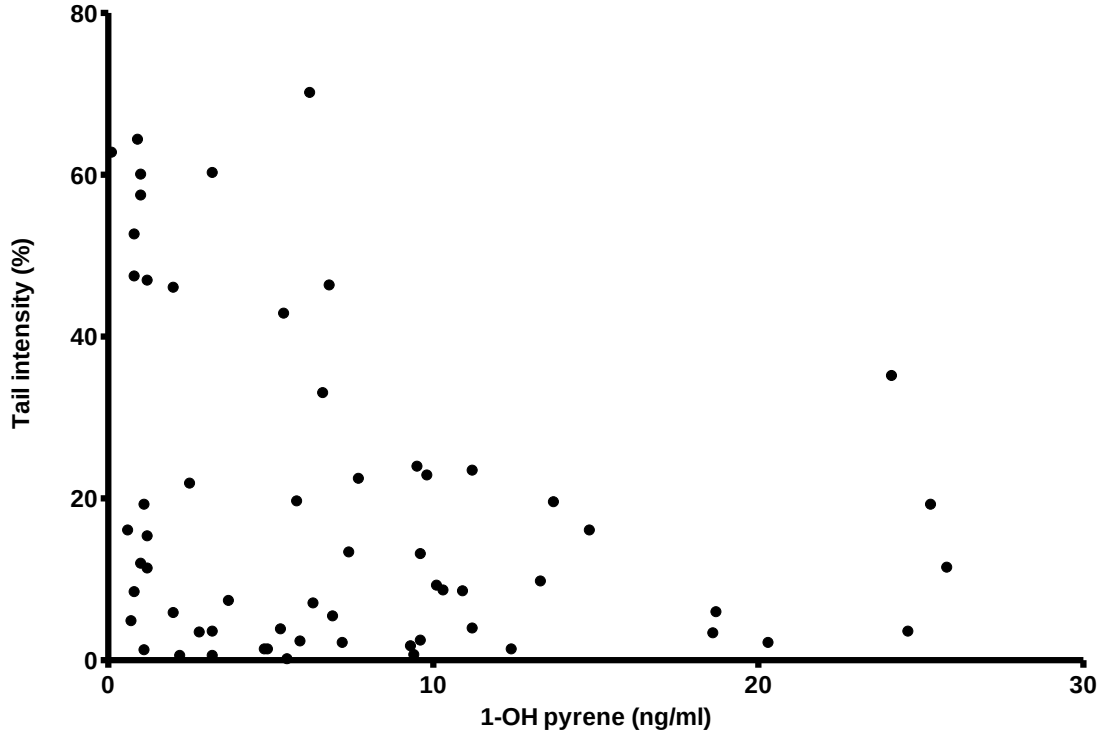

Figure 3.5. Dab correlation scatterplot with median tail intensity (dependent variable), and 1-hydroxypyrene in bile (independent variable).

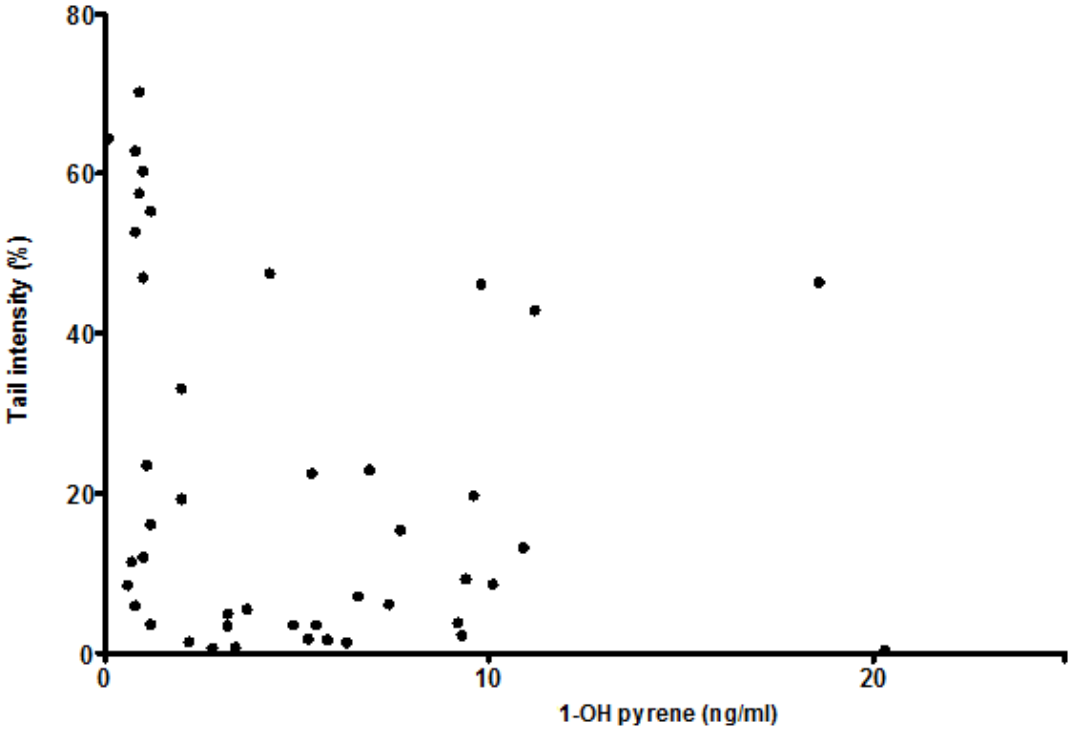

Figure 3.6. Haddock correlation scatterplot with median tail intensity (dependent variable), and 1-hydroxypyrene in bile (independent variable).

4. Discussion

4.1 Method development

There were higher levels of DNA damage in dab samples with gradual freezing compared to samples with direct immersion in lysis buffer. This was in particular the case for samples from Iceland 1, Iceland 2 and the Dogger Bank, which had 8-16 times higher levels of DNA damage with the gradual freezing technique compared to direct immersion in lysis buffer. The results were less clear for haddock. Haddock from the Firth of Forth had four times higher levels of DNA damage with gradual freezing compared to samples with direct immersion in lysis buffer. Haddock from Iceland had four times higher levels of DNA damage with direct immersion in lysis buffer compared to samples with gradual freezing.

Elevated levels of DNA damage confirm previously documented challenges of developing a suitable protocol for freezing of red blood cells prior to Comet assay (Doyle et al. 1988; Belpaeme et al. 1998). The passive freezing device ('Mr. Frosty') was used to reduce the cooling rate and prevent formation of ice crystals inside the cells. Ice crystals could cause considerable mechanical and physical damage and occur if the rate of cooling is too high. Similar damage could be induced due to osmotic stress (Grout et al. 1990). In line with standard freezing protocols, high freezing solution concentrations of Dimethyl sulfoxide (DMSO) were used to prevent this kind of damage. DMSO is a permeating agent which prevent ice crystal formation due to the binding of water (Doyle et al. 1988). DNA damage may also be caused by oxidation during storage and adding DMSO to the lysing buffer can prevent oxidative damage (Brunborg, pers. comm.). If cell membranes are damaged, release of unspecific endonucleases could quickly degrade DNA. In line with standards for the Comet assay, high solution concentrations of EDTA were used to prevent his kind of damage. EDTA is an anticoagulant which blocks the activity of several enzymes by chelating metal ions (Ca) (Kumar and Satchidanandam 2000). Enzymes not dependent on these cofactors could possibly induce DNA damage in red blood cells after thawing.

Low levels of DNA damage in dab samples from the reference areas indicate that direct immersion and prolonged storage (5 weeks) in lysis buffer is an acceptable technique for storage of red blood cells prior to Comet analysis. The Comet assay commonly involves a step of immersion in lysis buffer with the time perspective of hours (Collins 2004; Brunborg

2009). Studies on cultivated fish with immersion in lysis buffer for a prolonged period have indicated possible, but no clear change in Comet patterns (Belpaeme et al. 1998). To my knowledge this is the first successful study of Comet with samples taken on board a research vessel.

4.2 Genotoxicity in red blood cells

In accordance with quality standards for the Comet assay samples of dab from the Egersund bank were not used due to high background fluorescence. Samples from the Baltic Sea were only stored with the gradual freezing technique and therefore not acceptable for further analysis. Comet assay samples of haddock from the Egersund bank were not used due to high nucleoid density. Other parameters for the Egersund Bank and the Baltic Sea is consequently not discussed.

Female dab from the German Bight had significantly higher LSI compared to female dab from the other stations. Male haddock from Iceland 2 had significantly lower LSI compared to male haddock from the other stations. These differences could be due to food availability and composition, or exposure to chemicals. Most reports show a positive correlation between LSI and concentrations of chemicals (van der Oost et al. 2003), but these interactions are complex. Female dab from Iceland 2 had significantly lower GSI compared to female dab from the other stations. Female haddock from the Firth of Forth had significantly higher GSI compared to female haddock from the other stations. In general all GSI data are low, and this can be attributed to the post-spawning recovery which lasts from the end of August to the end of September (Filina et al. 2009). Further analysis were not separated between sexes due to unequal sex distribution for several stations, and exceptional sex differences in GSI and LSI.

The Comet assay has been scarcely used on red blood cells in natural populations of marine fish. The lifespan of fish red blood cells varies among species, but a range of 270-500 days has been reported (Avery et al. 1992; Goanvec et al. 2008). Damaged red blood cells tend to be removed at higher rates compared to undamaged red blood cells (Deflora et al. 1993). The detected damage (Comet) is the result of the equilibrium between this elimination and new formation of damaged cells.

The distance between the designated ICON stations could be considered satisfactory for regional comparisons. In addition cruises were carried out in the autumn which is suitable with respect to migration phases of the indicator species. Migrations, often in connection with spawning season, could result in masking of regional differences by intermixing of fish between stations (Damm et al. 1991).

4.2.1 The North Sea and Iceland

Dab from the Firth of Forth and Ekofisk in the North Sea had significantly higher levels of DNA damage compared to dab from the reference stations (Iceland). Dab from the Firth of Forth and Ekofisk had 14-18 and 6-8 times higher median levels of DNA damage compared to dab from Iceland 1 and Iceland 2, respectively. The German Bight and the Dan Field also had what appeared to be higher levels of DNA damage compared to both reference stations, but these differences were not statistically significant. There was no significant difference in levels of DNA damage in dab from the Dogger Bank compared to dab from Iceland.

As part of the ICON project, micronucleus assay has been carried out on the same dab blood as analyzed with the Comet assay. These data fit in with the results from the Comet assay (Baršienė, pers. comm.). Other micronucleus studies with dab have reported increased genotoxic effects in areas close to offshore activity (Ekofisk and the Danfield) and large rivers in the North Sea (the German Bight and the Firth of Forth) (Rybakovas et al. 2007).

Haddock from the Firth of Forth showed significantly lower levels of DNA damage compared to dab from Iceland 1. There was no significant difference in levels of DNA damage in haddock from the Firth of Forth compared to haddock from Iceland 2. Cod (*Gadus morhua*) has been shown to be less sensitive to action of some genotoxins compared to dab (Rybakovas et al. 2007). This could be the case for haddock, which is also a cod fish. Fish that live in polluted environments could in some cases develop a higher tolerance to pollutants. This tolerance is caused by mutations in the Ah receptor, resulting in decreased sensitivity of ligand binding (Nacci et al. 1999; Nacci 2002). The high levels of DNA damage for haddock from Iceland 1 are, however, difficult to assess and haddock may be sensitive to factors of which we do not have adequate knowledge.

PAHs are metabolized in the liver of fish and excreted to the bile as conjugated compounds. The rates of metabolism of PAH in fish are high, and the content of PAH metabolites in the bile shows good correlation with exposure to PAHs, both in relation to dose and the length of exposure (Collier and Varanasi 1991; Brumley et al. 1998). Some PAH-metabolites are known genotoxic substances (La Rocca et al. 1996) while others produce free radicals that lead to oxidative stress (Flowers-Geary et al. 1996). Offshore concentrations of PAHs in the North Sea are not always detectable, with exception for areas with extensive shipping and

offshore installations (OSPAR 2000). Atmospheric inputs of PAHs derived from fossile fuels and incineration processes is evident in coastal waters (Mastral 2003; Hartmann et al. 2004), but the estimates of such discharges are uncertain. Concentrations of PCBs and PAHs in areas around Iceland are the lowest reported for any oceanographic region (Schulz-Bull et al. 1998).

4.2.2 Coastal and offshore

Dab from the Firth of Forth and Ekofisk showed significantly higher levels of DNA damage compared to dab from the Dogger Bank. Dab from the Firth of Forth and Ekofisk had fourteen and six times higher levels of DNA damage, respectively compared to the Dogger Bank. The German Bight and the Dan Field also showed intermediate levels of DNA damage between levels of the Firth of Forth/Ekofisk, and the Dogger Bank, but these differences were not statistically significant. It was not possible to assess haddock DNA damage in the North Sea.

The highest levels of PAH sediment contamination have previously been reported at Ekofisk (35.87 ng /g dry weight) and the Firth of Forth (27.47 ng/g), followed by Danfield (13.78 ng/g). Lower levels have been reported for the German Bight (6.02 ng/g) and the Dogger Bank (5.79 ng/g) (Kammann et al. 2001).

Elevated levels of DNA damage in dab from the Firth of Forth indicates clear effects and probably reflects the historical chemical inputs from upstream industrial activity. The concentrations of persistent chemicals in this area are at levels that have been shown to pose a risk of effects on marine organisms. Similar concentrations have been found in a number of other coastal areas around industrial estuaries, including the German Bight (OSPAR 2010).

Elevated levels of DNA damage in dab from the offshore field of Ekofisk, suggests elevated concentrations of oil related substances. This effect was less clear for the Dan Field. During production of offshore oil and gas, water trapped in the reservoir is extracted with the oil or gas and becomes produced water. This fluid contains hydrocarbons and metals as well as chemicals added in the production process (Brendehaug et al. 1992). PAHs and alkylphenols contribute to a substantial part of the chemicals released into the environment. PAH metabolites in cod and PAHs in the soft tissue of mussels have shown exposure to effluents (Hylland et al. 2008). Some reports suggests that some alkylphenols also could contribute to oxidative stress in fish (Hasselberg et al. 2004). Exposure to some oestrogens have been

shown to reduce the activity of enzymes responsible for metabolism of most organic chemicals (CYP1A). This could also apply for alkylphenols as some of these substances have oestrogenic effect (Soto et al. 1991; Jobling and Sumpter 1993), and therefore could reduce the metabolism of chemicals. In general the amount of produced water released from a platform increases total production time (age) of the oil field, which then should be approximately equal in the case of Ekofisk and the Dan Field. Variation in the exact composition of produced water could be a source of variation between the two stations.

4.2.3 PAH exposure

The effect of most chemicals on aquatic organisms are still unknown (Ankley et al. 1996). Analysed contents of 1-hydroxypyrene (1-OH pyrene) was compared against the Comet response due to the expectation that genotoxic PAH metabolites would correlate with tail DNA. The contents of 1-OH pyrene in bile is often used as marker for the total metabolism of PAH compounds (Krahn et al. 1987; Ariese et al. 1993). The relationship between 1-OH pyrene in bile and concentration of PAHs in sediments is proportional and positively correlated (Hosnedl et al. 2003). Quantification of 1-OH pyrene is a good indication of PAH exposure in the last week before sampling. The scatterplots are not clear, but tends to a negative correlation between the compared variables. This observation could be due to limited data and inter-individual variability. The isolated data of 1-OH pyrene indicates higher levels of the PAH metabolite in bile of dab from the Dan Field, Ekofisk, the German Bight and the Firth of Forth compared to dab from the reference stations and Dogger Bank. This fits with the isolated total Comet response data already discussed. The isolated 1-OH pyrene data indicate low levels of PAH metabolites in bile of haddock for Iceland 1, Iceland 2 and the Firth of Forth.

4.2.4 Oxidative stress

Samples treated with the enzyme treatment procedure had higher tail DNA median values compared to the reference films, but reasonable comparisons were not possible due to the elevated levels of DNA damage with gradual freezing and storage prior to Comet analysis. The modified Comet assay protocol, which target oxidized DNA bases through the enzyme treatment procedure, has previously been shown to clearly enhance the sensitivity and specificity of the assay (Collins et al. 1993).

5. Conclusion

What was assumed to be baseline DNA damage was observed with direct immersion and storage in lysis buffer. The examined technique with gradual freezing did not contribute to a stable preservation of red blood cells of the examined species. The technique with direct immersion in lysis buffer and storage seems to be an acceptable method with respect to the preservation of red blood cells for subsequent analysis.

Results from the Comet assay clearly indicated that dab from coastal polluted areas (Firth of Forth) and offshore areas with nearby oil and gas activity (Ekofisk) had more DNA damage than fish from less polluted (Iceland) or offshore areas without nearby activity (Dogger Bank). The results were less clear for haddock, which could be due to species differences in response to chemicals.

The modified Comet assay seems to have worked, but it was not possible to identify specific DNA lesions caused by oxidative stress in red blood cells of dab and haddock based on available data.

The results clearly support Comet analyses of fish red blood cells as a useful parameter by which to assess environmental stress caused by chemicals, however, care needs to be taken at all steps in sample preparation and analyses.

6. Future work

Further testing of protocols with respect to both freezing techniques and cryo-preservation, and direct immersion and storage in lysis buffer of fish red blood cells should be carried out.

The use of other cells (i.e. white blood cells, gill cells) and other tissue should be assessed.

Increased knowledge of natural factors affecting the Comet response and species differences with respect to seasonality, disease and spawning should be assessed.

Specific DNA lesions caused by oxidative stress in red blood cells of fish species should be included for all samples.

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Appendix A: Raw data and analytical data

Dab

Station	#	Sex	Age	TI	Length	Body wt	Liver wt	Gonad wt	LSI	GSI	1-OH PYR	1-OH PHE	3-OH BAP
IS1	1	M	5	10.5	26	158	2.1	0.4	1.3	0.3	13.7	3.0	1.4
IS1	2	M	6	14.6	26	187	2.9	0.2	1.6	0.1	.	.	.
IS1	3	F	3	6.7	21	79	1.3	0.6	1.6	0.8	10.8	2.2	1.0
IS1	4	M	3	4.6	22	102	1.3	0.5	1.3	0.5	6.2	0.8	0.9
IS1	5	F	5	9.2	26	165	2.5	1.9	1.5	1.2	11.1	3.4	1.5
IS1	6	M	3	2.0	21	97	0.8	0.5	0.8	0.5	.	.	.
IS1	7	M	4	2.8	26	167	1.7	0.9	1.0	0.5	.	.	.
IS1	8	F	2	1.8	22	99	1.0	0.5	1.0	0.5	.	.	.
IS1	9	M	4	1.4	25	163	2.3	0.5	1.4	0.3	2.9	2.3	1.5
IS1	10	M	4	16.0	24	141	3.0	0.4	2.1	0.3	.	.	.
IS1	11	F	3	3.3	22	115	2.0	1.5	1.7	1.3	10.0	3.4	0.6
IS1	12	F	3	0.7	22	108	1.1	1.3	1.0	1.2	.	.	.
IS1	13	F	3	13.1	23	99	1.2	1.4	1.2	1.4	.	.	.
IS1	14	F	3	9.5	24	137	2.2	1.8	1.6	1.3	.	.	.
IS1	15	F	2	1.1	22	100	1.5	0.9	1.5	0.9	.	.	.
IS1	16	M	3	1.0	24	132	2.2	0.6	1.7	0.5	6.2	1.8	1.2
IS1	17	F	3	1.0	24	134	1.5	1.3	1.1	1.0	5.8	0.2	1.3
IS1	18	M	4	0.6	25	146	1.5	0.5	1.0	0.3	.	.	.
IS1	19	F	3	2.0	25	160	2.0	1.1	1.3	0.7	5.9	2.1	1.0
IS1	20	F	5	5.0	26	186	2.9	1.8	1.6	1.0	2.0	.	0.3
IS1	21	F	3	0.4	23	107	1.5	1.3	1.4	1.2	.	.	.
IS1	22	F	3	0.9	25	153	1.7	2.0	1.1	1.3	9.5	1.5	1.2
IS1	23	F	3	0.7	24	120	1.1	1.2	0.9	1.0	.	.	.
IS1	24	F	2	1.4	22	99	1.4	0.9	1.4	0.9	.	.	.
IS1	25	F	4	0.6	25	179	3.0	1.5	1.7	0.8	.	.	.
IS2	1	M	3	1.6	22	123	2.2	0.6	1.8	0.5	.	.	.
IS2	2	F	2	2.1	21	93	0.8	0.3	0.9	0.3	.	.	.
IS2	3	F	4	2.2	24	153	2.2	1.7	1.4	1.1	24.4	3.7	4.6
IS2	4	F	2	2.7	21	97	0.9	0.5	0.9	0.5	.	.	.
IS2	5	F	4	2.6	26	194	2.9	1.1	1.5	0.6	3.9	.	1.4
IS2	6	F	2	1.2	22	121	2.0	0.8	1.7	0.7	.	.	.
IS2	7	F	3	2.4	22	116	1.6	0.5	1.4	0.4	.	.	.
IS2	8	M	3	1.7	22	117	0.9	0.8	0.8	0.7	.	.	.
IS2	9	F	4	1.3	25	164	1.6	0.9	1.0	0.5	.	.	.
IS2	10	F	4	1.4	25	162	1.4	0.8	0.9	0.5	7.7	1.3	0.5
IS2	11	F	4	1.2	26	183	3.2	1.4	1.7	0.8	9.4	.	1.3
IS2	12	F	5	1.5	26	193	3.0	1.2	1.6	0.6	4.4	.	0.2
IS2	13	M	3	1.2	22	117	1.6	0.2	1.4	0.2	12.3	.	1.1
IS2	14	M	3	1.1	22	106	1.0	0.1	0.9	0.1	5.2	.	1.4
IS2	15	M	3	0.5	22	119	0.9	0.2	0.8	0.2	10.3	1.3	1.1
IS2	16	M	2	1.6	21	91	0.8	0.2	0.9	0.2	.	.	.
IS2	17	F	2	1.6	21	102	1.4	0.5	1.4	0.5	.	.	.
IS2	18	F	2	1.6	22	114	1.3	0.6	1.1	0.5	.	.	.
IS2	19	F	4	1.5	24	146	1.5	0.9	1.0	0.6	.	.	.
IS2	20	F	3	1.8	22	116	0.9	0.8	0.8	0.7	10.5	.	1.8
IS2	21	M	3	0.7	24	174	2.8	0.5	1.6	0.3	.	.	.
IS2	22	F	6	1.0	26	197	2.2	1.2	1.1	0.6	11.9	0.5	0.9

IS2	23	F	4	0.9	24	145	1.2	0.7	0.8	0.5	9.2	.	0.7
IS2	24	M	4	1.8	26	173	2.7	0.8	1.6	0.5	.	.	.
IS2	25	M	3	1.9	23	139	2.1	0.4	1.5	0.3	.	.	.
FF	1	F	3	17.8	22	92	0.9	1.3	1.0	1.4	.	.	.
FF	2	F	3	32.2	22	126	1.2	0.6	1.0	0.5	5.4	.	1.5
FF	3	F	4	25.3	23	123	1.8	1.8	1.5	1.5	11.8	0.7	1.5
FF	4	M	3	21.4	21	96	1.0	0.4	1.0	0.4	.	.	.
FF	5	F	4	50.5	22	127	2.3	1.8	1.8	1.4	5.4	.	2.0
FF	6	F	3	37.1	23	140	1.6	1.7	1.1	1.2	4.7	.	1.1
FF	7	F	3	35.4	22	113	1.3	1.2	1.2	1.1	.	.	.
FF	8	F	3	23.0	22	89	1.0	1.1	1.1	1.2	30.3	4.6	4.6
FF	9	F	3	43.1	22	121	2.3	1.2	1.9	1.0	10.1	.	1.3
FF	10	F	4	32.7	21	93	1.6	0.7	1.7	0.8	.	.	.
FF	11	F	3	35.7	20	83	0.7	1.0	0.8	1.2	.	.	.
FF	12	M	2	30.0	20	85	1.3	0.6	1.5	0.7	.	.	.
FF	13	M	2	21.9	21	89	2.0	0.8	2.2	0.9	.	.	.
FF	14	F	2	36.7	20	85	1.9	0.8	2.2	0.9	.	.	.
FF	15	F	2	32.4	21	97	1.7	0.4	1.8	0.4	.	.	.
FF	16	F	4	84.4	24	143	3.8	1.3	2.7	0.9	.	.	.
FF	17	F	4	27.8	24	146	1.6	2.0	1.1	1.4	.	.	.
FF	18	M	2	9.3	21	92	1.2	0.6	1.3	0.7	.	.	.
FF	19	F	2	.	21	88	1.1	0.8	1.3	0.9	.	.	.
FF	20	F	3	22.9	20	92	1.7	0.8	1.8	0.9	13.0	3.3	1.7
FF	21	F	2	8.3	22	111	1.1	1.6	1.0	1.4	.	.	.
FF	22	F	2	26.7	22	100	1.3	0.9	1.3	0.9	.	.	.
FF	23	F	3	13.8	22	107	1.2	1.4	1.1	1.3	11.8	.	1.8
FF	24	F	3	16.4	22	111	1.7	1.0	1.5	0.9	.	.	.
FF	25	M	2	31.5	21	91	1.3	0.6	1.4	0.7	.	.	.
GB	1	F	2	9.3	21	88	1.3	0.6	1.5	0.7	.	.	.
GB	2	F	2	11.0	22	108	2.1	1.2	1.9	1.1	.	.	.
GB	3	F	3	6.7	23	112	2.3	1.5	2.1	1.3	.	.	.
GB	4	F	2	12.3	21	119	3.5	1.4	2.9	1.2	.	.	.
GB	5	F	2	7.6	23	123	1.7	1.1	1.4	0.9	.	.	.
GB	6	F	3	7.6	24	159	4.9	1.5	3.1	0.9	.	.	.
GB	7	F	2	11.5	20	91	2.2	1.1	2.4	1.2	.	.	.
GB	8	F	1	9.0	20	87	2.6	1.1	3.0	1.3	.	.	.
GB	9	F	2	8.8	20	92	3.3	1.2	3.6	1.3	.	.	.
GB	10	F	2	23.2	20	90	2.7	0.9	3.0	1.0	.	.	.
GB	11	F	2	2.1	22	112	1.7	1.1	1.5	1.0	.	.	.
GB	12	F	4	1.3	25	143	2.0	1.7	1.4	1.2	12.8	1.9	1.0
GB	13	F	2	6.4	20	77	1.5	1.1	1.9	1.4	.	.	.
GB	14	F	2	10.3	21	103	3.4	0.8	3.3	0.8	.	.	.
GB	15	F	2	5.1	20	88	2.1	0.9	2.4	1.0	.	.	.
GB	16	F	3	7.6	24	89	3.2	0.9	3.6	1.0	.	.	.
GB	17	F	3	1.9	23	134	2.2	1.7	1.6	1.3	.	.	.
GB	18	F	3	1.8	23	116	0.9	1.0	0.8	0.9	.	.	.
GB	19	F	2	1.1	22	117	2.1	1.1	1.8	0.9	.	.	.
GB	20	F	2	2.7	21	107	1.0	1.6	0.9	1.5	.	.	.
GB	21	F	3	2.2	24	155	2.5	1.3	1.6	0.8	.	.	.
GB	22	M	2	0.8	20	.	1.2	0.2	.	.	.	.	.
GB	23	F	4	0.5	25	152	3.4	1.5	2.2	1.0	.	.	.
GB	24	F	3	0.9	22	118	1.8	1.0	1.5	0.8	16.8	0.6	1.6
GB	25	F	3	0.4	21	101	1.9	1.2	1.9	1.2	.	.	.
BA	1	M	2	.	23	132	1.4	2.4	1.1	1.8	.	.	.

BA	2	M	4	.	25	170	.	4.2	.	2.5	.	.	.
BA	3	M	3	.	24	128	1.2	3.8	0.9	3.0	.	.	.
BA	4	F	3	.	23	121	1.0	0.8	0.8	0.7	.	.	.
BA	5	F	2	.	23	118	1.0	1.2	0.8	1.0	.	.	.
BA	6	M	2	.	24	160	1.7	2.2	1.1	1.4	.	.	.
BA	7	F	2	.	23	144	2.6	2.0	1.8	1.4	.	.	.
BA	8	F	2	.	25	163	4.0	6.0	2.5	3.7	.	.	.
BA	9	M	3	.	23	130	2.0	1.5	1.5	1.2	.	.	.
BA	10	M	2	.	23	110	1.3	2.5	1.2	2.3	.	.	.
BA	11	F	2	.	23	150	2.1	2.6	1.4	1.7	.	.	.
BA	12	M	2	.	24	147	1.9	2.4	1.3	1.6	.	.	.
BA	13	F	2	.	25	144	2.2	2.6	1.5	1.8	.	.	.
BA	14	M	3	.	25	156	1.5	2.2	1.0	1.4	.	.	.
BA	15	M	3	.	24	148	0.9	0.1	0.6	0.1	.	.	.
BA	16	F	2	.	24	134	1.3	0.8	1.0	0.6	.	.	.
BA	17	M	2	.	23	131	1.5	2.3	1.1	1.8	.	.	.
BA	18	F	2	.	22	116	1.7	3.0	1.5	2.6	.	.	.
BA	19	M	2	.	22	107	0.7	2.1	0.7	2.0	.	.	.
BA	20	F	2	.	24	164	3.7	6.4	2.3	3.9	.	.	.
BA	21	F	2	.	22	119	2.1	5.5	1.8	4.6	.	.	.
BA	22	F	3	.	25	150	1.5	2.4	1.0	1.6	.	.	.
BA	23	F	2	.	23	141	3.2	2.5	2.3	1.8	.	.	.
BA	24	F	2	.	23	138	2.3	1.6	1.7	1.2	.	.	.
BA	25	F	2	.	24	146	2.8	3.9	1.9	2.7	.	.	.
DB	1	F	3	2.2	23	133	2.0	1.4	1.5	1.1	.	.	.
DB	2	F	2	3.5	21	99	1.1	1.1	1.1	1.1	.	.	.
DB	3	F	3	2.0	23	144	3.1	1.7	2.2	1.2	.	.	.
DB	4	F	3	2.5	22	114	2.0	1.0	1.8	0.9	9.6	0.7	1.7
DB	5	F	3	1.6	21	94	1.2	0.9	1.3	1.0	.	.	.
DB	6	F	2	.	21	110	2.0	1.2	1.8	1.1	.	.	.
DB	7	F	2	1.2	20	92	1.3	0.9	1.4	1.0	.	.	.
DB	8	F	2	1.2	21	91	0.8	0.6	0.9	0.7	.	.	.
DB	9	F	2	1.6	20	78	1.0	0.7	1.3	0.9	.	.	.
DB	10	F	3	.	20	71	0.6	0.6	0.8	0.8	.	.	.
DB	11	F	2	.	22	106	1.2	1.2	1.1	1.1	7.4	3.3	1.6
DB	12	F	2	2.8	23	123	1.9	1.3	1.5	1.1	.	.	.
DB	13	F	2	1.4	24	141	2.3	1.0	1.6	0.7	4.8	2.0	1.1
DB	14	F	2	3.3	20	77	0.9	0.6	1.2	0.8	.	.	.
DB	15	F	4	.	24	162	3.4	1.4	2.1	0.9	.	.	.
DB	16	F	3	.	21	84	0.7	0.8	0.8	1.0	.	.	.
DB	17	F	3	2.4	22	125	2.2	1.1	1.8	0.9	5.9	1.3	0.2
DB	18	F	3	2.2	23	116	1.0	1.6	0.9	1.4	7.2	.	1.7
DB	19	F	3	.	24	114	1.3	1.1	1.1	1.0	.	.	.
DB	20	F	3	.	22	122	1.6	0.9	1.3	0.7	.	.	.
DB	21	F	3	.	21	112	2.1	0.8	1.9	0.7	.	.	.
DB	22	F	2	.	22	116	1.7	1.2	1.5	1.0	8.9	1.3	0.9
DB	23	F	3	0.7	23	133	2.3	1.3	1.7	1.0	.	.	.
DB	24	F	2	1.3	21	90	0.7	1.0	0.8	1.1	.	.	.
DB	25	F	5	.	24	151	1.1	1.4	0.7	0.9	.	.	.
EB	1	F	5	.	24	113	.	1.2	.	1.1	.	.	.
EB	2	F	4	.	22	91	1.0	1.4	1.1	1.5	.	.	.
EB	3	F	5	.	21	88	1.4	1.5	1.6	1.7	.	.	.
EB	4	M	3	.	22	85	1.0	0.9	1.2	1.1	.	.	.
EB	5	F	4	.	22	109	1.8	1.4	1.7	1.3	.	.	.

EB	6	M	3	.	21	80	0.7	1.0	0.9	1.2	.	.	.
EB	7	F	6	.	22	80	1.2	1.1	1.5	1.4	.	.	.
EB	8	F	3	.	23	111	1.6	1.4	1.4	1.3	.	.	.
EB	9	F	5	.	21	83	0.6	1.2	0.7	1.5	.	.	.
EB	10	F	5	.	21	91	1.5	1.8	1.7	2.0	.	.	.
EB	11	F	5	.	21	78	1.1	1.4	1.4	1.8	.	.	.
EB	12	F	5	.	22	78	0.4	0.5	0.5	0.6	.	.	.
EB	13	M	5	.	22	103	0.8	0.8	0.8	0.8	.	.	.
EB	14	M	4	.	20	75	0.9	1.2	1.2	1.6	.	.	.
EB	15	M	3	.	21	77	1.7	1.1	2.2	1.4	.	.	.
EB	16	F	6	.	23	104	0.9	1.1	0.9	1.1	.	.	.
EB	17	M	3	.	20	73	1.1	1.1	1.5	1.5	.	.	.
EB	18	M	4	.	22	94	1.1	3.3	1.2	3.5	.	.	.
EB	19	F	3	.	22	96	1.1	1.3	1.1	1.4	.	.	.
EB	20	F	4	.	24	138	2.0	2.0	1.4	1.4	.	.	.
EB	21	F	3	.	23	98	0.7	1.2	0.7	1.2	.	.	.
EB	22	M	4	.	21	86	1.1	1.1	1.3	1.3	.	.	.
EB	23	M	4	.	21	87	1.2	1.4	1.4	1.6	.	.	.
EB	24	M	4	.	21	82	1.9	1.3	2.3	1.6	.	.	.
EB	25	M	4	.	21	84	1.0	1.6	1.2	1.9	.	.	.
P01	1	F	4	3.6	23	117	.	.	.	.	24.6	0.7	2.9
P01	2	F	4	19.3	23	125	.	.	.	.	25.3	0.0	2.7
P01	3	F	3	6.0	23	121	.	.	.	.	18.7	0.2	1.7
P01	4	F	3	.	20	79	.	.	.	.	.	.	.
P01	5	F	3	.	22	103	.	.	.	.	10.8	1.6	1.5
P01	6	F	3	3.5	23	116	.	.	.	.	.	.	.
P01	7	F	2	7.7	20	83	.	.	.	.	.	.	.
P01	8	M	4	6.3	24	160	.	.	.	.	.	.	.
P01	9	M	3	7.9	23	105	.	.	.	.	.	.	.
P01	10	M	2	5.1	22	106	.	.	.	.	.	.	.
P01	11	M	3	1.7	24	140	.	.	.	.	.	.	.
P01	12	M	3	2.9	23	131	.	.	.	.	.	.	.
P01	13	M	2	3.1	21	98	.	.	.	.	.	.	.
P01	14	M	3	4.5	21	82	.	.	.	.	.	.	.
P01	15	F	6	3.0	30	263	.	.	.	.	.	.	.
P01	16	F	4	.	25	163	.	.	.	.	.	.	.
P01	17	F	4	2.4	25	144	.	.	.	.	.	.	.
P01	18	F	5	2.1	26	185	.	.	.	.	.	.	.
P01	19	M	4	2.4	24	133	.	.	.	.	.	.	.
P01	20	M	2	.	20	76	.	.	.	.	.	.	.
P01	21	M	3	.	23	112	.	.	.	.	.	.	.
P01	22	M	3	.	20	83	.	.	.	.	.	.	.
P01	23	M	2	1.2	21	90	.	.	.	.	.	.	.
P01	24	M	2	0.8	20	83	.	.	.	.	.	.	.
P01	25	M	3	.	21	90	.	.	.	.	.	.	.
EF	1	F	2	19.5	20	85	1.4	1.2	1.6	1.4	.	.	.
EF	2	F	2	11.7	21	99	2.0	1.2	2.0	1.2	.	.	.
EF	3	F	3	16.1	21	89	1.1	0.9	1.2	1.0	14.8	.	1.5
EF	4	F	3	10.6	21	86	1.7	1.2	2.0	1.4	.	.	.
EF	5	F	3	14.5	25	158	1.6	1.8	1.0	1.1	.	.	.
EF	6	F	4	21.9	24	124	0.8	1.6	0.6	1.3	2.5	.	1.0
EF	7	F	3	19.6	23	103	1.3	1.0	1.3	1.0	13.7	.	1.5
EF	8	F	4	21.4	23	121	2.1	1.6	1.7	1.3	.	.	.
EF	9	F	2	19.1	23	118	1.4	1.3	1.2	1.1	.	.	.

EF	10	F	2	24.0	21	94	1.1	1.2	1.2	1.3	9.5	0.2	1.2
EF	11	F	2	11.5	21	88	0.9	0.8	1.0	0.9	25.8	.	2.9
EF	12	F	2	35.2	20	83	1.2	1.4	1.4	1.7	24.1	.	2.5
EF	13	F	3	17.3	20	74	0.9	1.0	1.2	1.4	.	.	.
EF	14	F	2	25.1	21	92	1.2	0.9	1.3	1.0	.	.	.
EF	15	F	3	9.8	23	95	0.9	0.7	0.9	0.7	13.3	.	1.9
EF	16	F	3	8.7	24	132	1.4	1.7	1.1	1.3	10.3	.	1.2
EF	17	F	3	11.1	21	86	1.6	1.7	1.9	2.0	.	.	.
EF	18	F	3	2.9	23	117	1.1	2.0	0.9	1.7	.	.	.
EF	19	F	3	4.0	22	103	1.5	1.0	1.5	1.0	11.2	0.1	1.4
EF	20	M	3	12.1	24	130	1.8	0.5	1.4	0.4	.	.	.
EF	21	F	3	.	24	153	1.8	1.4	1.2	0.9	15.6	0.2	1.8
EF	22	M	2	1.9	21	123	0.8	0.2	0.7	0.2	.	.	.
EF	23	F	3	3.5	20	80	1.5	1.4	1.9	1.8	.	.	.
EF	24	F	3	1.4	23	143	1.6	1.6	1.1	1.1	12.4	0.2	1.6
EF	25	F	3	1.9	25	150	2.8	1.5	1.9	1.0	.	.	.

Haddock

Station	#	Sex	Age	TI	Length	Body wt	Liver wt	Gonad wt	LSI	GSI	1-OH PYR	1-OH PHE	3-OH BAP
IS1	1	F	3	.	38	551	28.0	2.6	5.1	0.5	.	.	.
IS1	2	F	3	46.4	38	578	21.5	2.8	3.7	0.5	6.8	.	1.8
IS1	3	F	3	3.4	36	424	10	2.4	2.4	0.6	18.6	0.5	1.6
IS1	4	M	3	60.3	33	368	12.7	0.4	3.5	0.1	3.2	.	1.3
IS1	5	M	3	60.1	38	506	18.2	0.8	3.6	0.2	1.0	.	0.3
IS1	6	M	3	71.8	34	301	5	0.7	1.7	0.2	.	.	.
IS1	7	M	3	55.3	35	393	12.5	0.8	3.2	0.2	.	.	.
IS1	8	M	3	47.0	34	394	12.1	0.3	3.1	0.1	1.2	.	0.2
IS1	9	M	3	12.0	39	610	33.9	0.4	5.6	0.1	1.0	.	.
IS1	10	F	3	57.5	40	668	37.1	2.5	5.6	0.4	1.0	.	.
IS1	11	M	3	.	38	570	36.3	0.4	6.4	0.1	0.9	0.0	0.0
IS1	12	M	3	70.2	40	664	37	0.6	5.6	0.1	6.2	0.5	.
IS1	13	M	3	64.4	39	545	29.6	1.2	5.4	0.2	0.9	.	.
IS1	14	F	3	62.8	42	735	34.7	4.3	4.7	0.6	0.1	.	0.2
IS1	15	F	3	47.5	40	630	35.7	2.6	5.7	0.4	0.8	.	.
IS1	16	M	3	.	37	462	15.3	0.5	3.3	0.1	4.3	1.6	1.4
IS1	17	M	3	1.3	39	519	16.2	0.4	3.1	0.1	1.1	.	.
IS1	18	M	6	7.1	45	866	39.4	1.3	4.5	0.2	6.3	0.6	0.6
IS1	19	M	6	33.1	39	586	18	0.6	3.1	0.1	6.6	0.3	2.7
IS1	20	M	5	5.9	36	518	30.2	1.2	5.8	0.2	2.0	.	1.6
IS1	21	F	3	52.7	41	662	27.4	2.5	4.1	0.4	0.8	.	1.1
IS1	22	F	4	8.5	41	698	22.9	2.8	3.3	0.4	0.8	.	0.2
IS1	23	M	4	16.1	44	822	46.3	0.4	5.6	0.0	0.6	.	.
IS1	24	F	3	11.4	41	798	46.3	3.5	5.8	0.4	1.2	.	1.3
IS1	25	M	3	4.9	45	876	34.5	0.6	3.9	0.1	0.7	.	.
IS2	1	M	5	3.6	44	789	23	1.1	2.9	0.1	3.2	.	1.4
IS2	2	M	5	15.4	40	620	15.6	0.6	2.5	0.1	1.2	.	1.2
IS2	3	M	4	22.5	38	497	6.8	0.5	1.4	0.1	7.7	.	1.5
IS2	4	F	4	42.9	42	725	51.6	4	7.1	0.6	5.4	.	1.8
IS2	5	M	3	23.5	37	520	16.8	0.5	3.2	0.1	11.2	.	2.5
IS2	6	M	5	19.3	42	703	20.4	1	2.9	0.1	1.1	.	1.0
IS2	7	M	2	46.1	36	508	31	0.4	6.1	0.1	2.0	.	1.0

IS2	8	M	5	22.9	39	484	4	0.5	0.8	0.1	9.8	.	1.8
IS2	9	F	5	5.5	40	588	13.5	3.1	2.3	0.5	6.9	.	0.8
IS2	10	M	4	7.4	38	543	18.5	0.5	3.4	0.1	3.7	.	0.7
IS2	11	F	5	1.7	37	496	11.2	2.3	2.3	0.5	.	.	.
IS2	12	F	2	19.7	32	288	2.6	0.9	0.9	0.3	5.8	.	2.5
IS2	13	F	2	13.2	35	438	39.9	1.6	9.1	0.4	9.6	.	2.1
IS2	14	M	2	8.6	32	374	14.2	0.2	3.8	0.1	10.9	1.9	2.6
IS2	15	M	3	9.3	36	470	7.5	0.6	1.6	0.1	10.1	1.3	1.7
IS2	16	F	2	0.7	33	340	5.2	1	1.5	0.3	9.4	0.4	2.3
IS2	17	F	2	0.3	32	305	3.6	0.9	1.2	0.3	.	.	.
IS2	18	M	2	2.2	33	338	8.2	0.2	2.4	0.1	20.3	2.0	3.1
IS2	19	F	3	1.8	37	476	16.5	2	3.5	0.4	9.3	.	0.8
IS2	20	F	3	3.9	34	482	12.4	1.8	2.6	0.4	5.3	.	1.9
IS2	21	M	2	.	30	266	3.2	0.3	1.2	0.1	.	.	.
IS2	22	M	2	0.6	37	491	20.7	0.5	4.2	0.1	3.2	.	1.2
IS2	23	M	2	3.5	32	320	3.7	0.2	1.2	0.1	2.8	.	1.1
IS2	24	M	2	1.4	33	368	11.2	0.4	3.0	0.1	4.9	.	1.1
IS2	25	F	6	0.6	41	655	13.6	3.2	2.1	0.5	2.2	.	0.3
FF	1	M	3	13.9	27	176	3.8	0.3	2.2	0.2	.	.	.
FF	2	M	3	6.6	26	193	8	0.6	4.1	0.3	.	.	.
FF	3	F	3	6.1	33	330	11.7	1.9	3.5	0.6	.	.	.
FF	4	F	3	13.4	33	399	20.8	2.8	5.2	0.7	7.4	0.7	1.7
FF	5	F	1	27.3	30	278	17.1	1.5	6.2	0.5	.	.	.
FF	6	M	1	3.1	27	204	11.9	0.4	5.8	0.2	.	.	.
FF	7	F	2	5.3	25	205	9.4	0.8	4.6	0.4	.	.	.
FF	8	M	2	0.8	25	186	8.2	0.5	4.4	0.3	.	.	.
FF	9	M	1	1.0	27	201	12	0.4	6.0	0.2	.	.	.
FF	10	M	3	3.8	28	226	9.1	0.4	4.0	0.2	.	.	.
FF	11	F	3	.	28	253	12.2	1.5	4.8	0.6	9.2	0.0	2.0
FF	12	M	3	1.6	31	309	19.9	0.4	6.4	0.1	.	.	.
FF	13	F	3	4.3	27	214	8.7	1.6	4.1	0.7	.	.	.
FF	14	F	2	1.1	28	222	12.5	1.3	5.6	0.6	.	.	.
FF	15	M	2	2.5	25	142	3.4	0.3	2.4	0.2	.	.	.
FF	16	M	3	.	29	235	9.4	0.2	4.0	0.1	.	.	.
FF	17	M	3	0.6	26	171	4.9	0.5	2.9	0.3	.	.	.
FF	18	F	2	0.8	33	341	20.7	2.3	6.1	0.7	.	.	.
FF	19	F	2	0.8	31	275	10.4	1.5	3.8	0.5	.	.	.
FF	20	M	3	0.7	30	293	17.2	0.8	5.9	0.3	.	.	.
FF	21	F	3	.	34	349	9.6	2.5	2.8	0.7	3.4	0.0	1.3
FF	22	F	3	.	32	289	12.3	1.4	4.3	0.5	6.6	0.0	1.4
FF	23	M	3	3.5	29	239	10.5	0.4	4.4	0.2	.	.	.
FF	24	F	3	0.2	37	523	14.4	3.5	2.8	0.7	5.5	.	1.6
FF	25	M	2	.	30	300	22.6	0.7	7.5	0.2	.	.	.
EB	1	F	1	.	28	183	9.2	0.5	5.0	0.3	.	.	.
EB	2	M	1	.	27	161	11.5	<0.1	7.1		.	.	.
EB	3	F	1	.	31	250	21.8	1	8.7	0.4	.	.	.
EB	4	M	2	.	32	292	12.7	2.5	4.4	0.9	.	.	.
EB	5	F	1	.	31	243	19.1	1.7	7.9	0.7	.	.	.
EB	6	F	1	.	28	205	12	0.6	5.9	0.3	.	.	.
EB	7	M	1	.	28	211	10.4	0.7	4.9	0.3	.	.	.
EB	8	M	1	.	29	234	13.4	0.8	5.7	0.3	.	.	.
EB	9	M	1	.	32	288	15.7	0.4	5.5	0.1	.	.	.
EB	10	M	1	.	27	178	9.9	0.2	5.6	0.1	.	.	.
EB	11	M	1	.	30	233	14.9	1.2	6.4	0.5	.	.	.

EB	12	M	1	.	27	198	10.1	0.8	5.1	0.4	.	.	.
EB	13	M	3	.	38	503	28.5	3.5	5.7	0.7	.	.	.
EB	14	M	1	.	32	346	16.6	0.7	4.8	0.2	.	.	.
EB	15	F	1	.	31	277	17	0.7	6.1	0.3	.	.	.
EB	16	F	1	.	31	256	20.9	1.2	8.2	0.5	.	.	.
EB	17	F	1	.	30	211	15.4	0.8	7.3	0.4	.	.	.
EB	18	F	1	.	32	294	25.8	1.6	8.8	0.5	.	.	.
EB	19	M	1	.	30	254	14.2	1.3	5.6	0.5	.	.	.
EB	20	M	3	.	39	555	34.6	4.5	6.2	0.8	.	.	.
EB	21	M	3	.	41	652	51.1	7.2	7.8	1.1	.	.	.
EB	22	M	1	.	32	347	20.0	0.8	5.8	0.2	.	.	.
EB	23	M	3	.	38	495	26.4	3.5	5.3	0.7	.	.	.
EB	24	M	1	.	30	257	16.1	0.8	6.3	0.3	.	.	.
EB	25	M	1	.	29	244	9.7	0.3	4.0	0.1	.	.	.

Age: year, TI: tail intensity, length: cm, weight: g, liver weight; g, gonad weight: g, LSI: %, GSI: %, 1-OH pyrene, 1-OH phenanthrene and 3-OH benzo(a)pyrene: ng/ml

Appendix B: Solutions and media

PBS without Ca, Mg

0.200 g KH_2PO_4

0.200 g KCl

2.902 g $Na_2HPO_412H_2O$

8.000 g NaCl

Dissolved in dH_2O, amounting a total volume of 1.000 l and adjusted to pH 7.4. Osmolality should be approximately 280 mOsm/kg. Autoclaved for 30 minutes at 121 °C.

PBS for washing of sheep blood

1.450 g KH_2PO_4

7.600 g $Na_2HPO_412H_2O$

4.800 g NaCl

Dissolved in dH_2O, amounting a total volume of 1.000 l and adjusted to pH 7.4. Osmolality should be approximately 270 mOsm/kg. Autoclaved for 30 minutes at 121 °C.

PBS without Ca, Mg + EDTA (10 mM)

1.86 g EDTA

Dissolved in 500 ml PBS without Ca and Mg, adjusted to pH 7.4 with NaOH.

0.75% softagar (low melting point)

0.075 g NuSieve® GTG® Agarose

Heated in 10 ml PBS without Ca, Mg + EDTA (10 mM) to boiling point until dissolving of Agarose and kept at 37 °C (warming block).

Lysis stock solution

730.5 g NaCl (2.5 M)

186.0 g EDTA (100 mM)

6.0 g Tris-base (10 mM)

Added to 3 l dH_2O under stirring and adjusted to approximately pH 10 with 37.5 g NaOH pellet before adding of 50 g SLS (1%). Stock left stirring until components was dissolved, new adjustment to pH 10 with concentrated HCl or NaOH.

Lysis buffer (four GelBond® films)

300 ml Lysis stock solution

33.3 ml DMSO (10%)

3.33 ml Triton-X (1%)

Enzyme reaction buffer (Collins buffer)

47.65 g Hepes (40 mM)

37.25 g KCl (0.1 M)

0.90 g EDTA (0.5 mM)

Dissolved in 4 l dH_2O and adjusted to pH 7.6 with KOH (7 M). Added dH_2O until total volume of 5 l.

Electrophoresis stock solution (X10)

600 g NaOH (10 M)

18.6 g EDTA (200 mM)

Dissolved in 4 l dH2O. Added dH_2O until total volume of 5 l.

Electrophoresis buffer

1.35 l dH2O + 0.15 l electrophoresis stock solution (X10).

Neutralising buffer

242.5 g Tris-base (0.4 M)

Dissolved in 4 l dH_2O and adjusted to pH 7.5 with concentrated HCl.

TE-buffer

5 ml EDTA (200 mM)

20 ml Tris-HCl (0,5 M)

Dissolved in 0.8 l dH_2O and adjusted to pH 8.0. Added dH_2O until total volume of 1 l.

Freezing solution

10 ml FCS (20%)

5 ml DMSO (10%)

RPMI + 1 l dH_2O

Appendix C: Products and manufacturers

Product	Producer	Country
Absolutt alkohol prima (100% ethanol)	Arcus	Norway
Dimethyl sulphoxide (DMSO)	Merck	Germany
Distilled water	Locally produced	Norway
Ethylenediaminetetraacetic acid (EDTA)	Sigma	USA
Foetal calf serum (FCS)	Gibco	USA
Fpg	Locally produced	Norway
GelBond® Film	Cambrex	USA
Hepes	Sigma	USA
Hydrogen chloride (HCl)	Merck	Germany
Isopropanol prima	Arcus	Norway
NuSieve® GTG® Agarose	Cambrex	USA
Phosphate buffer solution (PBS)	Locally produced	Norway
Potassium chloride (KCl)	Merck	Germany
Potassium dihydrogenphosphate (KH_2PO_4)	Merck	Germany
Potassium hydroxide (KOH)	Merck	Germany
Sodium chloride (NaCl)	Merck	Germany
Sodium hydrogenphosphate (Na_2HPO_4)	Merck	Germany
Sodium hydroxide (NaOH)	Merck	Germany
Sodium lauroyl sarcosinate (SLS)	Sigma	UK
SYBR® Gold	Invitrogen	USA
Trisma® HCl	Sigma	USA
Triton-X	Sigma	USA
Trizma® base	Sigma	USA